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Identification of virulence associated regions RD1 and RD5 enabling the development of improved vaccines of M. bovis BCG and M. microti

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Identification of virulence associated regions RD1 and RD5 leading to improve vaccine of *M. bovis* BCG and *M. microti*

Virulence associated regions are searched for a long time in *Mycobacterium*. The present invention concerns the identification of 2 genomic regions which are shown to be associated with a virulent phenotype in *Mycobacteria* and particularly in *M. tuberculosis* and in *M. leprae*. It concerns also the fragments of said regions.

The two regions are known as RD1 and RD5 as disclosed in Molecular Microbiology (1999), vol. 32, pages 643 to 655 (Gordon S.V. et al.). Both of these regions or at least one of them are absent from the vaccine strains of *M. bovis* BCG and in *M. microti*. strains found involved and used as live vaccines in the 1960's.

Other applications which are encompassed by the present invention are related to the use of all or part of the said regions to detect virulent strains of *Mycobacteria* and particularly *M. tuberculosis* in humans and animals. The RD1 and RD5 are considered as virulence markers under the present invention.

The recombinant Mycobacteria and particularly *M. bovis* BCG after modification of their genome by introduction of all part of RD1 region and/or RD5 region in said genome can

be used for the immune system of patients affected with a cancer as for example a bladder cancer.

The present invention relates to a strain of *M. bovis* BCG or *M. microti*, wherein said strain has integrated part or all of the RD1 region responsible for enhanced immunogenicity to the tubercle bacilli, especially the genes coding ESAT-6 and CFP-10 antigenes. These strains will be referred as the *M. bovis* BCG::RD1 or *M. microti*::RD1 strains and are useful as a new improved vaccine for prevention of tuberculosis infections and for treating superficial bladder cancer.

Mycobacterium bovis BCG (bacille Calmette-Guérin) has been used since 1921 to prevent tuberculosis although it is of limited efficacy against adult pulmonary disease in highly endemic areas. *Mycobacterium microti*, another member of the *Mycobacterium tuberculosis* complex, was originally described as the infective agent of a tuberculosis-like disease in voles (*Microtus agrestis*) in the 1930's (Wells, A. Q. 1937. Tuberculosis in wild voles. Lancet 1221 and Wells, A. Q. 1946. The murine type of tubercle bacillus. Medical Research council special report series 259:1-42.). Until recently, *M. microti* strains were thought to be pathogenic only for voles, but not for humans and some were even used as a live-vaccine. In fact, the vole bacillus proved to be safe and effective in preventing clinical tuberculosis in a trial involving roughly 10,000 adolescents in the UK in the 1950's (Hart, P. D. a., and I. Sutherland. 1977. BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life. British Medical Journal 2:293-295). At about the same time, another strain, OV166, was successfully administered to half a million newborns in Prague, former Czechoslovakia, without any serious complications (Sula, L., and I. Radkovsky. 1976. Protective effects of M. microti vaccine against tuberculosis. J. Hyg. Epid. Microbiol. Immunol. 20:1-6). *M. microti*

vaccination has since been discontinued because it was no more effective than the frequently employed BCG vaccine. As a result, improved vaccines are needed for preventing and treating tuberculosis.

The problem for attempting to ameliorate this live vaccine is that the molecular mechanism of both the attenuation and the immunogenicity of BCG is still poorly understood. Comparative genomic studies of all six members of the *M. tuberculosis* complex have identified more than 140 genes, whose presence is facultative, that may confer differences in phenotype, host range and virulence. Relative to the genome of the paradigm strain, *M. tuberculosis* H37Rv *(S. T. Cole, et al., Nature 393, 537 (1998))*, many of these genes occur in chromosomal regions that have been deleted from certain species (RD1-16, RvD1-5), M. A. Behr, et al., Science 284, 1520 (1999) ; R. Brosch, et al., Infection Immun. 66, 2221 (1998) ; S. V. Gordon, et al., Molec Microbiol 32, 643 (1999) ; H. Salamon, et al, Genome Res 10, 2044 (2000), G. G. Mahairas et al, J. Bacteriol. 178, 1274 (1996) and R. Brosch, et al., Proc Natl Acad Sci USA 99, 3684 (2002).

In connection with the invention and based on their distribution among tubercle bacilli and potential to encode virulence functions, RD1, RD3-5, RD7 and RD9 (Fig. 1A, B) were accorded highest priority for functional genomic analysis using "knock-ins" of *M. bovis* BCG to assess their potential contribution to the attenuation process. Clones spanning these RD regions were selected from an ordered *M. tuberculosis* H37Rv library of integrating shuttle cosmids *(*S. T. Cole, *et al.*, *Nature* **393**, 537 (1998) and W. R. Bange, et al, *Tuber. Lung Dis.* 79, 171 (1999)*)*, and individually electroporated into BCG Pasteur, where they inserted stably into the *attB* site *(*M. H. Lee, et al, *Proc. Natl. Acad. Sci. USA* **88**, 3111 (1991)*)*.

We have uncovered that only reintroduction of RD1 led to profound phenotypic alteration. Strikingly, the BCG::RD1 "knock-in" grew more vigorously than BCG

controls in immuno-deficient mice, inducing extensive splenomegaly and granuloma formation.

RD1 is restricted to the avirulent strains *M. bovis* BCG and *M. microti*. Although the endpoints are not identical, the deletions have removed from both vaccine strains a cluster of six genes (Rv3871-Rv3876) that are part of the ESAT-6 locus (Fig. 1A (S. T. Cole, *et al.*, *Nature* **393**, 537 (1998) and F. Tekaia, *et al.*, *Tubercle Lung Disease* **79**, 329 (1999)).

Among the missing products are members of the mycobacterial PE (Rv3872), PPE (Rv3873), and ESAT-6 (Rv3874, Rv3875) protein families. Despite lacking obvious secretion signals, ESAT-6 (Rv3875) and the related protein CFP-10 (Rv3874), are abundant components of short-term culture filtrate, acting as immunodominant T-cell antigens that induce potent Th1 responses (F. Tekaia, et al., Tubercle Lung Disease 79, 329 (1999) ; A. L. Sorensen, et al, Infect. Immun. 63, 1710 (1995) and R. Colangelli, et al., Infect. Immun. 68, 990 (2000)).

In summary, we have discovered that the restoration of RD1 to *M. bovis* BCG leads to increased persistence in immunocompetent mice. The *M. bovis* BCG::RD1 strain induces RD1-specific immune responses of the Th1-type, has enhanced immunogenicity and confers better protection than *M. bovis* BCG alone in the mouse model of tuberculosis. The *M. bovis* BCG::RD1 vaccine is significantly more virulent than *M. bovis* BCG in immunodeficient mice but considerably less virulent than *M. tuberculosis*.

In addition, we show that *M. microti* lacks a different but overlapping part of the RD1 region ($RD1^{mic}$) to *M. bovis* BCG and our results indicate that reintroduction of RD1 confers increased virulence of BCG ::RD1 in immunodeficient mice. The rare strains of *M. microti* that are associated with human disease contain a region referred to as $RD5^{mic}$ whereas those from voles do not.

M. bovis BCG vaccine could be improved by reintroducing other genes encoding ESAT-6 family members that have been lost, notably, those found in the RD8 and RD5 loci of *M. tuberculosis*. These regions also code for additional T-cell antigens.

M; bovis BCG::RD1 could be improved by reintroducing the RD8 and RD5 loci of M. tuberculosis.

M. bovis BCG vaccine could be improved by overexpressing the genes contained in the RD1, RD5 and RD8 regions.

Accordingly, these new strains, showing greater persistence and enhanced immunogenicity, represent an improved vaccine for preventing tuberculosis and treating bladder cancer.

In addition, the greater persistence of these recombinant stains is an advantage for the presentation of other antigens, for instance from HIV in humans and in order to induce protection immune responses. Those improved strains may also be of use in veterinary medicine, for instance in preventing bovine tuberculosis.

Description

Therefore, the present invention is aimed at a strain of *M. bovis* BCG or *M. microti*, wherein said strain has integrated all or part of the RD1 region responsible for enhanced immunogenicity to the tubercle bacilli. These strains will be referred as the *M. bovis* BCG::RD1 or *M. microti*::RD1 strains.

In connection with the invention, "part or all of the RD1 region" means that the strain has integrated a portion of DNA originating from *Mycobacterium tuberculosis*, which

comprises at least one gene selected from Rv3871, Rv3872 (mycobacterial PE), Rv3873 (PPE), Rv3874 (CFP-10), Rv3875 (ESAT-6), and Rv3876. The expression gene is referred herein as the coding sequence in frame with its natural promoter as well as the coding sequence which has been isolated and framed with an exogenous promoter, for example a promoter capable of directing high level of expression of said coding sequence.

In a specific aspect, the invention relates to a strain of *M. bovis* BCG or *M. microti* wherein said strain has integrated at least one gene selected from Rv3871, Rv3872 (SEQ ID No 2, mycobacterial PE), Rv3873 (SEQ ID No 3, PPE), Rv3874 (SEQ ID No 4, CFP-10), Rv3875 (SEQ ID No 5, ESAT-6), and Rv3876, preferably CFP-10, ESAT-6 or both (SEQ ID No 6).

These genes can be mutated (deletion, insertion or base modification) so as to maintain the improved immunogenicity while decreasing the virulence of the strains. Using routine procedure, the man skilled in the art can select the *M. bovis* BCG::RD1 or *M. microti*::RD1 strains, in which a mutated gene has been integrated, showing improved immunogenicity and lower virulence.

We have shown here that introduction of the RD1 region makes the vaccine strains induce a more effective immune response against a challenge with *M. tuberculosis*. However, this first generation of constructs can be followed by other, more fine-tuned generations of constructs as the complemented BCG::RD1 vaccine strain also showed a more virulent phenotype in severely immuno-compromised (SCID) mice. Therefore, the BCG RD1+ constructs may be modified to as to be applicable as vaccine strains while being safe for immuno-compromised individuals.

In this perspective, the man skilled in the art can adapt the BCG::RD1 strain by the conception of BCG vaccine strains that only carry parts of the genes coding for ESAT-6 or CFP-10 in a mycobacterial expression vector (for example pSM81) under the control

of a promoter, more particularly an hsp60 promoter. For example, at least one portion of the esat-6 gene that codes for immunogenic 20-mer peptides of ESAT-6 active as T-cell epitopes (Mustafa AS, Oftung F, Amoudy HA, Madi NM, Abal AT, Shaban F, Rosen Krands I, & Andersen P. (2000) Multiple epitopes from the Mycobacterium tuberculosis ESAT-6 antigen are recognized by antigen-specific human T cell lines. Clin Infect Dis. 30 Suppl 3:S201-5, peptides P1 to P8 are incorporated herein in the descritption) could be cloned into this vector and electroporated into BCG, resulting in a BCG strain that produces these epitopes.

Alternatively, the ESAT-6 and CFP-10 encoding genes (for example on plasmid RD1-AP34 and or RD1-2F9) could be altered by directed mutagenesis (using for example QuikChange Site-Directed Mutagenesis Kit from Stratagen) in a way that most of the immunogenic peptides of ESAT-6 remain intact, but the biological functionality of ESAT-6 is lost.
This approach could result in a more protective BCG vaccine without increasing the virulence of the recombinant BCG construct.

Therefore, the invention is also aimed at a method for preparing and selecting *M. bovis* BCG or *M. microti* strains comprising a step consisting of modifying the *M. bovis* BCG::DR1 or *M. microt*::DR1 strains as defined above by insertion, deletion or mutation in the integrated DR1 region, more particularly in the esat-6 or CFP-10 gene, said method leading to strains that are less virulent for immuno-depressed individuals.
Together, these methods would allow to explain what causes the effect that we see with our BCG::RD1 strain (the presence of additional T-cell epitopes from ESAT-6 and CFP10 resulting in increased immunogenicity) or whether the effect is caused by better fitness of the recombinant BCG::RD1 clones resulting in longer exposure time of the immune system to the vaccine - or - by a combinatorial effect of both factors.

In a preferred embodiment, the invention is aimed at the *M. bovis* BCG::RD1 strains, which have integrated a cosmid herein referred to as the RD1-2F9 and RD1-AP34 contained in the E. coli strains deposited on April 2, 2002 at the CNCM (Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris cedex 15, France) under the accession number I-2831 and I-2832 respectively. The RD1-2F9 is a cosmid comprising a portion of the *Mycobacterium tuberculosis* H37Rv genome that spans the RD1 region and the hygromycin resistance gene. The RD1-AP34 is a cosmid comprising a portion of the *Mycobacterium tuberculosis* DNA containing two genes coding for ESAT-6 and CFP-10 as well as a gene conferring resistance to Kanamycin.

The construct RD1-AP34 contains a 3909 bp fragment of the *M. tuberculosis* H37Rv genome from region 4350459 bp to 4354367 bp cloned into an integrating vector pKint (SEQ ID No 1). The Accession No. of the segment 160 of the *M. tuberculosis* H37Rv genome that contains this region is AL022120.

SEQ ID No 1 :

1 - gaattcccat ccagtgagtt caaggtcaag cggcgccccc ctggccaggc atttctcgtc
61 - tcgccagacg gcaaagaggt catccaggcc ccctacatcg agcctccaga agaagtgttc
121 - gcagcacccc caagcgccgg ttaagattat ttcattgccg gtgtagcagg acccgagctc
181 - agcccggtaa tcgagttcgg gcaatgctga ccatcgggtt tgtttccggc tataaccgaa
241 - cggtttgtgt acgggataca aatacaggga gggaagaagt aggcaa*atgg aaaaaatgtc*
301 - acatgatccg atcgctgccg acattggcac gcaagtgagc gacaacgctc tgcacggcgt
361 - gacggccggc tcgacggcgc tgacgtcggt gaccgggctg gttcccgcgg gggccgatga
421 - ggtctccgcc caagcggcga cggcgttcac atcggagggc atccaattgc tggcttccaa
481 - tgcatcggcc caagaccagc tccaccgtgc gggcgaagcg gtccaggacg tcgcccgcac
*541 - ctattcgcaa atcgacgacg gcgccgccgg cgtcttcgcc gaa*taggccc ccaacacatc
601 - ggagggagtg atcaccatgc tgtggcacgc aatgccaccg gagctaaata ccgcacggct

661 - gatggccggc gcgggtccgg ctccaatgct tgcggcggcc gcgggatggc agacgctttc

721 - ggcggctctg gacgctcagg ccgtcgagtt gaccgcgcgc ctgaactctc tgggagaagc

781 - ctggactgga ggtggcagcg acaaggcgct tgcggctgca acgccgatgg tggtctggct

841 - acaaaccgcg tcaacacagg ccaagacccg tgcgatgcag gcgacggcgc aagccgcggc

901 - atacacccag gccatggcca cgacgccgtc gctgccggag atcgccgcca accacatcac

961 - ccaggccgtc cttacggcca ccaacttctt cggtatcaac acgatcccga tcgcgttgac

1021 - cgagatggat tatttcatcc gtatgtggaa ccaggcagcc ctggcaatgg aggtctacca

1081 - ggccgagacc gcggttaaca cgcttttcga gaagctcgag ccgatggcgt cgatccttga

1141 - tcccggcgcg agccagagca cgacgaaccc gatcttcgga atgccctccc ctggcagctc

1201 - aacaccggtt ggccagttgc cgccggcggc tacccagacc ctcggccaac tgggtgagat

1261 - gagcggcccg atgcagcagc tgacccagcc gctgcagcag gtgacgtcgt tgttcagcca

1321 - ggtgggcggc accggcggcg gcaacccagc cgacgaggaa gccgcgcaga tgggcctgct

1381 - cggcaccagt ccgctgtcga accatccgct ggctggtgga tcaggcccca gcgcgggcgc

1441 - gggcctgctg cgcgcggagt cgctacctgg cgcaggtggg tcgttgaccc gcacgccgct

1501 - gatgtctcag ctgatcgaaa agccggttgc cccctcggtg atgccggcgg ctgctgccgg

1561 - atcgtcggcg acgggtggcg ccgctccggt gggtgcggga gcgatgggcc agggtgcgca

1621 - atccggcggc tccaccaggc cgggtctggt cgcgccggca ccgctcgcgc aggagcgtga

1681 - agaagacgac gaggacgact gggacgaaga ggacgactgg tgagctcccg taatgacaac

1741 - agacttcccg gccacccggg ccggaagact tgccaacatt ttggcgagga aggtaaagag

1801 - agaaagtagt ccagcatggc agagatgaag accgatgccg ctaccctcgc gcaggaggca

1861 - ggtaatttcg agcggatctc cggcgacctg aaaacccaga tcgaccaggt ggagtcgacg

1921 - gcaggttcgt tgcagggcca gtggcgcggc gcggcgggga cggccgccca ggccgcggtg

1981 - gtgcgcttcc aagaagcagc caataagcag aagcaggaac tcgacgagat ctcgacgaat

2041 - attcgtcagg ccggcgtcca atactcgagg gccgacgagg agcagcagca ggcgctgtcc

2101 - tcgcaaatgg gcttctgacc cgctaatacg aaaagaaacg gagcaaaaac *atgacagagc*

2161 - *agcagtggaa tttcgcgggt atcgaggccg cggcaagcgc aatccaggga aatgtcacgt*

2221 - *ccattcattc cctccttgac gaggggaagc agtccctgac caagctcgca gcggcctggg*

2281 - *gcggtagcgg ttcggaggcg taccagggtg tccagcaaaa atgggacgcc acggctaccg*

2341 - *agctgaacaa cgcgctgcag aacctggcgc ggacgatcag cgaagccggt caggcaatgg*

2401 - *cttcgaccga aggcaacgtc actgggatgt tcgca*taggg caacgccgag ttcgcgtaga

2461 - atagcgaaac acgggatcgg gcgagttcga ccttccgtcg gtctcgccct ttctcgtgtt

2521 - tatacgtttg agcgcactct gagaggttgt catggcggcc gactacgaca agctcttccg

2581 - gccgcacgaa ggtatggaag ctccggacga tatggcagcg cagccgttct tcgaccccag

2641 - tgcttcgttt ccgccggcgc ccgcatcggc aaacctaccg aagcccaacg gccagactcc

2701 - gcccccgacg tccgacgacc tgtcggagcg gttcgtgtcg gccccgccgc cgccaccccc

2761 - acccccacct ccgcctccgc caactccgat gccgatcgcc gcaggagagc cgccctcgcc

2821 - ggaaccggcc gcatctaaac cacccacacc ccccatgccc atcgccggac ccgaaccggc

2881 - cccacccaaa ccacccacac cccccatgcc catcgccgga cccgaaccgg ccccacccaa

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2941 - accacccaca cctccgatgc ccatcgccgg acctgcaccc accccaaccg aatcccagtt

3001 - ggcgcccccc agaccaccga caccacaaac gccaaccgga gcgccgcagc aaccggaatc

3061 - accggcgccc cacgtaccct cgcacgggcc acatcaaccc cggcgcaccg caccagcacc

3121 - gccctgggca aagatgccaa tcggcgaacc cccgcccgct ccgtccagac cgtctgcgtc

3181 - cccggccgaa ccaccgaccc ggcctgcccc ccaacactcc cgacgtgcgc gccggggtca

3241 - ccgctatcgc acagacaccg aacgaaacgt cgggaaggta gcaactggtc catccatcca

3301 - ggcgcggctg cgggcagagg aagcatccgg cgcgcagctc gccccggaa cggagccctc

3361 - gccagcgccg ttgggccaac cgagatcgta tctggctccg cccacccgcc ccgcgccgac

3421 - agaacctccc cccagccccc cgccgcagcg caactccggt cggcgtgccg agcgacgcgt

3481 - ccaccccgat ttagccgccc aacatgccgc ggcgcaacct gattcaatta cggccgcaac

3541 - cactggcggt cgtcgccgca agcgtgcagc gccggatctc gacgcgacac agaaatcctt

3601 - aaggccggcg gccaaggggc cgaaggtgaa gaaggtgaag ccccagaaac cgaaggccac

3661 - gaagccgccc aaagtggtgt cgcagcgcgg ctggcgacat tgggtgcatg cgttgacgcg

3721 - aatcaacctg ggcctgtcac ccgacgagaa gtacgagctg gacctgcacg ctcgagtccg

3781 - ccgcaatccc cgcgggtcgt atcagatcgc cgtcgtcggt ctcaaaggtg gggctggcaa

3841 - aaccacgctg acagcagcgt tggggtcgac gttggctcag gtgcgggccg accggatcct

3901 - ggctctaga

pos. 0001-0006 **EcoRI-restriction site**

pos. 0286-0583 *Rv3872 coding for a PE-Protein* (SEQ ID No 2)

pos. 0616-1720 Rv3873 coding for a PPE-Protein (SEQ ID No 3)

pos. 1816-2115 **Rv3874 coding for Culture Filtrat protein 10kD (CFP10)** (SEQ ID No 4)

pos. 2151-2435 *Rv3875 coding for Early Secreted Antigen Target 6kD (ESAT6)* (SEQ ID No 5)

pos. 3903-3609 **XbaI-restriction site**

pos. 1816-2435 CFP-10 gene + esat-6 gene (SEQ ID No 6)

The sequence of the fragment RD1-2F9 (~ 32 kb) covers the region of the M. tuberculosis genome AL123456 from ca 4337 kb to ca. 4369 kb, and also contains the sequence described above.

Such strains fulfill the aim of the invention which is to provide an improved tuberculosis vaccine or *M. bovis* BCG-based prophylactic or therapeutic agent, or a recombinant *M. microti* derivative for these purposes.

The above described *M. bovis* BCG::RD1 strains are better tuberculosis vaccines than *M. bovis* BCG. These strains can also be improved by reintroducing other genes found in the RD8 and RD5 loci of *M. tuberculosis.* These regions code for additional T-cell antigens. As indicated, overexpressing the genes contained in the RD1, RD5 and RD8 regions by means of exogenous promoters is encompassed by the invention. The same applies regarding *M. microti*::RD1 strains. *M. microti* strains could also be improved by reintroducing the RD8 locus of *M. tuberculosis.*

In a second embodiment, the invention is directed to a cosmid or a plasmid comprising part or all of the RD1 region originating from *Mycobacterium tuberculosis*, said region

comprising at least one gene selected from Rv3871, Rv3872 (mycobacterial PE), Rv3873 (PPE), Rv3874 (CFP-10), Rv3875 (ESAT-6), and Rv3876. Preferably, such cosmids or plasmid comprises CFP-10, ESAT-6 or both. The invention also relates to the use of these cosmids or plasmids for transforming *M. bovis* BCG or *M. microti* strains. As indicated above, these cosmids or plasmids may comprises a mutated gene selected from Rv3871 to Rv3876, said mutated gene being responsible for the improved immunogenicity and decreased virulence.

In another embodiment, the invention embraces a pharmaceutical composition comprising a strain as depicted above and a pharmaceutically acceptable carrier.

In addition to the strains, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the living vaccine into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

Preferably, such composition is suitable for oral intravenous or subcutaneous administration.

The determination of the effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient, i.e the number of strains administered, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit large therapeutic indices

are preferred. Of course, ED50 is to be modulated according to the mammal to be treated or vaccinated. In this regard, the invention contemplates a composition suitable for human administration as well as veterinary composition.

The invention is also aimed at a vaccine comprising a *M. bovis* BCG::RD1 or *M. microti*::RD1 strain as depicted above and a suitable carrier. This vaccine is especially useful for preventing tuberculosis. It can also be used for treating bladder cancer.

The invention also concerns a product comprising a strain as depicted above and at least one protein selected from ESAT-6 and CFP-10 or epitope derived thereof for a separate, simultaneous or sequential use for treating tuberculosis.

In still another embodiment, the invention concerns the use of a *M. bovis* BCG::RD1 or *M. microti*::RD1 strain as depicted above for preventing or treating tuberculosis. It also concerns the use of a *M. bovis* BCG::RD1 or *M. microti*::RD1 strain as a powerful adjuvant/immunomodulator used in the treatment of superficial bladder cancer.

The invention also contemplates the identification at the species level of members of the *M. tuberculosis* complex by means of an RD-based molecular diagnostic test. Inclusion of markers for $RD1^{mic}$ and $RD5^{mic}$ would improve the tests and act as predictors of virulence, especially in humans. In this regard, the invention concerns a diagnostic kit comprising DNA probes and primers specifically hybridizing to a DNA portion of the RD1 or RD5 region, more particularly probes hybridizing under stringent conditions to a gene selected from Rv3871, Rv3872 (mycobacterial PE), Rv3873 (PPE), Rv3874 (CFP-10), Rv3875 (ESAT-6), and Rv3876, preferably CFP-10 and ESAT-6. As used herein, the term "stringent conditions" refers to conditions which permit hybridization between the probe sequences and the polynucleotide sequence to be detected. Suitably stringent conditions can be defined by, for example, the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and

are well known in the art. In particular, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature. The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Variations on the above ranges and conditions are well known in the art.

Among the preferred primers, we can cite:
primer esat-6F GTCACGTCCATTCATTCCCT (SEQ ID No 9),
primer esat-6R ATCCCAGTGACGTTGCCTT) (SEQ ID No 10),
primer RD1^{mic} flanking region F GCAGTGCAAAGGTGCAGATA (SEQ ID No 11),
primer RD1^{mic} flanking region R GATTGAGACACTTGCCACGA (SEQ ID No 12),
primer RD5^{mic} flanking region F GAATGCCGACGTCATATCG (SEQ ID No 16),
primer RD5^{mic} flanking region R CGGCCACTGAGTTCGATTAT (SEQ ID No 17).

The present invention covers also the complentary nucleotidic sequences of said above primers as well as the nucleotidic sequences hybridizing under stringent conditions with them and having at least 20 nucleotides and less than 500 nucleotides.

Diagnostic kits for the identification at the species level of members of the *M. tuberculosis* comprising antibodies directed to mycobacterial PE, PPE, CFP-10 and ESAT-6 are also embraced by the invention. As used herein, the term "antibody" refers to intact molecules as well as fragments thereof, such as Fab, F(ab').sub.2, and Fv, which are capable of binding the epitopic determinant. Probes or antibodies can be labeled with isotopes, fluorescent or phosphorescent molecules or by any other means known in the art.

The invention is further detailed below and will be illustrated with the following figures.

Figure legends

Figure 1: *M. bovis* BCG and *M. microti* have a chromosomal deletion, RD1, spanning the *cfp10-esat6* locus.

(A) Map of the *cfp10-esat6* region showing the six possible reading frames and the *M. tuberculosis* H37Rv gene predictions. This map is also available at: (http://genolist.pasteur.fr/TubercuList/).

The deleted regions are shown for BCG (red) and *M. microti* (blue) with their respective H37Rv genome coordinates, and the extent of the conserved ESAT-6 locus (F. Tekaia, *et al.*, *Tubercle Lung Disease* **79**, 329 (1999)), is indicated by the gray bar.

(B) Table showing characteristics of deleted regions selected for complementation analysis. Potential virulence factors and their putative functions disrupted by each deletion are shown. The coordinates are for the *M. tuberculosis* H37Rv genome.

(C) Clones used to complement BCG. Individual clones spanning RD1 regions (RD1-I106 and RD1-2F9) were selected from an ordered *M. tuberculosis* genomic library (R.B. unpublished) in pYUB412 (S. T. Cole, *et al.*, *Nature* **393**, 537 (1998) and W. R. Bange, F. M. Collins, W. R. Jacobs, Jr., *Tuber. Lung Dis.* **79**, 171 (1999)) and electroporated into *M. bovis* BCG strains, or *M. microti*. Hygromycin-resistant transformants were verified using PCR specific for the corresponding genes. pAP35 was derived from RD1-2F9 by excision of an *Afl*II fragment. pAP34 was constructed by subcloning an *Eco*RI-*Xba*I fragment into the integrative vector pKINT. The ends of each fragment are related to the BCG RD1 deletion (shaded box) with black lines and the H37Rv coordinates for the other fragment ends given in kilobases.

(D) Immunoblot analysis, using an ESAT-6 monoclonal antibody, of whole cell protein extracts from log-phase cultures of H37Rv (S. T. Cole, *et al.*, *Nature* **393**, 537 (1998)),

BCG::pYUB412 (M. A. Behr, *et al.*, *Science* **284**, 1520 (1999)), BCG::RD1-I106 (R. Brosch, *et al.*, *Infection Immun.* **66**, 2221 (1998)), BCG::RD1-2F9 (S. V. Gordon, *et al.*, *Molec Microbiol* **32**, 643 (1999)), *M. bovis* (H. Salamon et al, *Genome Res* **10**, 2044 (2000)), *Mycobacterium smegmatis* (G. G. Mahairas, et al, *J. Bacteriol.* **178**, 1274 (1996)), *M. smegmatis*::pYUB412, and *M. smegmatis*:: RD1-2F9 (R. Brosch, *et al.*, *Proc Natl Acad Sci USA* **99**, 3684 (2002)).

Figure 2: Complementation of BCG Pasteur with the RD1 region alters the colony morphology and leads to accumulation of Rv3873 and ESAT-6 in the cell wall.

(A) Serial dilutions of 3 week old cultures of BCG::pYUB412, BCG::I106 or BCG::RD1-2F9 growing on Middlebrook 7H10 agar plates. The white square shows the area of the plate magnified in the image to the right.

(B) Light microscope image at fifty fold magnification of BCG::pYUB412 and BCG::RD1-2F9 colonies. 5 µl drops of bacterial suspensions of each strain were spotted adjacently onto 7H10 plates and imaged after 10 days growth, illuminating the colonies through the agar.

(C) Immunoblot analysis of different cell fractions of H37Rv obtained from http://www.cvmbs.colostate.edu/microbiology/tb/ResearchMA.html using either an anti-ESAT-6 antibody or

(D) anti-Rv3873 (PPE) rabbit serum. H37Rv and BCG signify whole cell extracts from the respective bacteria and Cyt, Mem and CW correspond to the cytosolic, membrane and cell wall fractions of *M. tuberculosis* H37Rv.

Figure 3: Complementation of BCG Pasteur with the RD1 region increases bacterial persistence and pathogenicity in mice.

(A) Bacteria in the spleen and lungs of BALB/c mice following intravenous (i.v.) infection via the lateral tail vein with 10^6 colony forming units (cfu) of *M. tuberculosis* H37Rv (red) or 10^7 cfu of either BCG::pYUB412 (yellow) or BCG::RD1-I106 (green). **(B)** Bacterial persistence in the spleen and lungs of C57BL/6 mice following i.v. infection with 10^5 cfu of BCG::pYUB412 (yellow), BCG::RD1-I106 (green) or BCG::RD1-2F9 (blue).

(C) Bacterial multiplication after i.v. infection with 10^6 cfu of BCG::pYUB412 (yellow) and BCG::RD1-2F9 (blue) in severe combined immunodeficiency mice (SCID). For **A**, **B**, and **C** each timepoint is the mean of 3 to 4 mice and the error bars represent standard deviations.

(D) Spleens from SCID mice three weeks after i.v. infection with 10^6 cfu of either BCG::pYUB412, BCG::RD1-2F9 or BCG::I301 (an RD3 "knock-in", Fig. 1B). The scale is in cm.

Figure 4: Immunisation of mice with BCG::RD1 generates marked ESAT-6 specific T-cell responses and enhanced protection to a challenge with *M. tuberculosis*.

(A) Proliferative response of splenocytes of C57BL/6 mice immunised subcutaneously (s.c.) with 10^6 CFU of BCG::pYUB412 (open squares) or BCG::RD1-2F9 (solid squares) to *in vitro* stimulation with various concentrations of synthetic peptides from poliovirus type 1 capsid protein VP1, ESAT-6 or Ag85A (K. Huygen, et al., *Infect. Immun.* **62**, 363 (1994), L. Brandt, *J.Immunol.* **157**, 3527 (1996) and C. Leclerc et al, *J. Virol.* **65**, 711 (1991)).

(B) Proliferation of splenocytes from BCG::RD1-2F9-immunised mice in the absence or presence of 10 μg/ml of ESAT-6 1-20 peptide, with or without 1 μg/ml of anti-CD4

(GK1.5) or anti-CD8 (H35-17-2) monoclonal antibody. Results are expressed as mean and standard deviation of ^{3}H-thymidine incorporation from duplicate wells.

(C) Concentration of IFN-γ in culture supernatants of splenocytes of C57BL/6 mice stimulated for 72 h with peptides or PPD after s.c. or i.v. immunisation with either BCG::pYUB412 (red and yellow) or BCG::RD1-2F9 (green and blue). Mice were inoculated with either 10^6 (yellow and green) or 10^7(red and blue) cfu. Levels of IFN-γ were quantified using a sandwich ELISA (detection limit of 500 pg/ml) with the mAbs R4-6A2 and biotin-conjugated XMG1.2. Results are expressed as the mean and standard deviation of duplicate culture wells.

(D) Bacterial counts in the spleen and lungs of vaccinated and unvaccinated BALB/c mice 2 months after an i.v. challenge with *M. tuberculosis* H37Rv. The mice were challenged 2 months after i.v. inoculation with 10^6 cfu of either BCG::pYUB412 or BCG::RD1-2F9. Organ homogenates for bacterial enumeration were plated on 7H11 medium, with or without hygromycin, to differentiate *M. tuberculosis* from residual BCG colonies. Results are expressed as the mean and standard deviation of 4 to 5 mice and the levels of significance derived using the Wilcoxon rang sum test.

Figure 5: *Mycobacterium microti* strain OV254 BAC map (named MiXXX), overlaid on the *M. tuberculosis* H37Rv (named RvXXX) and *M. bovis* AF2122/97 (named MbXXX) BAC maps. The scale bars indicate the position on *the M. tuberculosis* genome.

Figure 6: Difference in the region 4340-4360 kb between the deletion in BCG RD1^{bcg} (A) and in *M. microti* RD1^{mic} (C) relatively to *M. tuberculosis* H37Rv (B).

Figure 7: Difference in the region 3121-3127 kb between *M. tuberculosis* H37Rv (A) and *M. microti* OV254 (B). Gray boxes picture the direct repeats (DR), black ones the unique numbered spacer sequences. * spacer sequence identical to the one of spacer 58 reported by van Embden *et al.* (42). Note that spacers 33-36 and 20-22 are not shown because H37Rv lacks these spacers.

Figure 8: **A)** *Ase*I PFGE profiles of various *M. microti* strains; Hybridization with a radiolabeled **B)** *esat-6* probe; **C)** probe of the $RD1^{mic}$ flanking region; **D)** *plcA* probe. 1. *M. bovis* AF2122/97, 2. *M. canetti*, 3. *M. bovis* BCG Pasteur, 4. *M. tuberculosis* H37Rv, 5. *M. microti* OV254, 6. *M. microti* Myc 94-2272, 7. *M. microti* B3 type mouse, 8. *M. microti* B4 type mouse , 9. *M. microti* B2 type llama, 10. *M. microti* B1 type llama, 11. *M. microti* ATCC 35782. M: Low range PFGE marker (NEB).

Figure 9: PCR products obtained from various *M. microti* strains using primers that flank the $RD1^{mic}$ region, for amplifying ESAT-6 antigen, that flank the MiD2 region. 1. *M. microti* B1 type llama, 2. *M. microti* B4 type mouse, 3. *M. microti* B3 type mouse, 4. *M. microti* B2 type llama, 5. *M. microti* ATCC 35782, 6. *M. microti* OV254, 7. *M. microti* Myc 94-2272, 8. *M. tuberculosis* H37Rv.

Example 1: preparation and assessment of *M. bovis* BCG::RD1 strains as a vaccine for treating or preventing tuberculosis.

As mentioned above, we have found that complementation with RD1 was accompanied by a change in colonial appearance as the BCG Pasteur "knock-in" strains developed a strikingly different morphotype (Fig. 2A). The RD1 complemented strains adopted a

spreading, less-rugose morphology, that is characteristic of *M. bovis,* and this was more apparent when the colonies were inspected by light microscopy (Fig. 2B). Maps of the clones used are shown (Fig. 1C). These changes were seen following complementation with all of the RD1 constructs (Fig. 1C) and on complementing *M. microti* (data not shown). Pertinently, Calmette and Guérin (A. Calmette, *La vaccination preventive contre la tuberculose.* (Masson et cie., Paris, 1927)) observed a change in colony morphology during their initial passaging of *M. bovis*, and our experiments now demonstrate that this change, corresponding to loss of RD1, directly contributed to attenuating this virulent strain. The integrity of the cell wall is known to be a key virulence determinant for *M. tuberculosis* (C. E. Barry, *Trends Microbiol* **9**, 237 (2001)), and changes in both cell wall lipids (M. S. Glickman, J. S. Cox, W. R. Jacobs, Jr., *Mol Cell* **5**, 717 (2000)) and protein (F. X. Berthet, *et al., Science* **282**, 759 (1998)) have been shown to alter colony morphology and diminish persistence in animal models.

To determine which genes were implicated in these morphological changes, antibodies recognising three RD1 proteins (Rv3873, CFP10 and ESAT-6) were used in immunocytological and subcellular fractionation analysis. When the different cell fractions from *M. tuberculosis* were immunoblotted all three proteins were localized in the cell wall fraction (Fig. 2C) though significant quantities of Rv3873, a PPE protein, were also detected in the membrane and cytosolic fractions (Fig. 2D). Using immunogold staining and electron microscopy the presence of ESAT-6 in the envelope of *M. tuberculosis* was confirmed but no alteration in capsular ultrastructure could be detected (data not shown). Previously, CFP-10 and ESAT-6 have been considered as secreted proteins (F. X. Berthet et al, *Microbiology* **144**, 3195 (1998)) but our results suggest that their biological functions are linked directly with the cell wall.

Changes in colonial morphology are often accompanied by altered bacterial virulence. Initial assessment of the growth of different BCG::RD1 "knock-ins" in C57BL/6 or BALB/c mice following intravenous infection revealed that complementation did not

restore levels of virulence to those of the reference strain *M. tuberculosis* H37Rv (Fig. 3A). In longer-term experiments, modest yet significant differences were detected in the persistence of the BCG::RD1 "knock-ins" in comparison to BCG controls. Following intravenous infection of C57BL/6 mice, only the RD1 "knock-ins" were still detectable in the lungs after 106 days (Fig. 3B). This difference in virulence between the RD1 recombinants and the BCG vector control was more pronounced in severe combined immunodeficiency (SCID) mice (Fig. 3C). The BCG::RD1 "knock-in" was markedly more virulent, as evidenced by the growth rate in lungs and spleen and also by an increased degree of splenomegaly (Fig. 3D). Cytological examination revealed numerous bacilli, extensive cellular infiltration and granuloma formation. These increases in virulence following complementation with the RD1 region, demonstrate that the loss of this genomic locus contributed to the attenuation of BCG.

The inability to restore full virulence to BCG Pasteur was not due to instability of our constructs nor to the strain used (data not shown). Essentially identical results were obtained on complementing BCG Russia, a strain less passaged than BCG Pasteur and presumed, therefore, to be closer to the original ancestor (M. A. Behr, *et al.*, *Science* **284**, 1520 (1999)). This indicates that the attenuation of BCG was a polymutational process and loss of residual virulence for animals was documented in the late 1920s (T. Oettinger, et al, *Tuber Lung Dis* **79**, 243 (1999)). Using the same experimental strategy, we also tested the effects of complementing with RD3-5, RD7 and RD9 (S. T. Cole, *et al., Nature 393, 537 (1998)* ; M. A. Behr, *et al., Science 284, 1520 (1999)* ; R. Brosch, *et al., Infection Immun. 66, 2221 (1998) and* S. V. Gordon *et al., Molec Microbiol 32, 643 (1999))* encoding putative virulence factors (Fig. 1B). Reintroduction of these regions, which are not restricted to avirulent strains, did not affect virulence in immuno-competent mice. Although it is possible that deletion effects act synergistically it seems more plausible that other attenuating mechanisms are at play.

Since RD1 encodes at least two potent T-cell antigens (R. Colangelli, *et al.*, *Infect. Immun.* **68**, 990 (2000), M. Harboe, et al., *Infect. Immun.* **66**, 717 (1998) and R. L. V. SkjØt, *et al.*, *Infect. Immun.* **68**, 214 (2000)), we investigated whether its restoration induced immune responses to these antigens or even improved the protective capacity of BCG. Three weeks following either intravenous or subcutaneous inoculation with BCG::RD1 or BCG controls, we observed similar proliferation of splenocytes to an Ag85A (an antigenic BCG protein) peptide (K. Huygen, et al., *Infect. Immun.* **62**, 363 (1994)), but not against a control viral peptide (Fig. 4A). Moreover, BCG::RD1 generated powerful $CD4^+$ T-cell responses against the ESAT-6 peptide as shown by splenocyte proliferation (Fig. 4A, B) and strong IFN-γ production (Fig. 4C). In contrast, the BCG::pYUB412 control did not stimulate ESAT-6 specific T-cell responses thus indicating that these were mediated by the RD1 locus. ESAT-6 is, therefore, highly immunogenic in mice in the context of recombinant BCG.

When used as a subunit vaccine, ESAT-6 elicits T-cell responses and induces levels of protection weaker than but akin to those of BCG (L. Brandt et al, *Infect. Immun.* **68**, 791 (2000)). Challenge experiments were conducted to determine if induction of immune responses to BCG::RD1-encoded antigens, such as ESAT-6, could improve protection against infection with *M. tuberculosis*. Groups of mice inoculated with either BCG::pYUB412 or BCG::RD1 were subsequently infected intravenously with *M. tuberculosis* H37Rv. These experiments showed that immunisation with the BCG::RD1 "knock-in" inhibited the growth of *M. tuberculosis* within both BALB/c (Fig. 4D) and C57BL/6 mice when compared to inoculation with BCG alone.

Although the increases in protection induced by BCG::RD1 and the BCG control are modest they demonstrate convincingly that genetic differences have developed between the live vaccine and the pathogen which have weakened the protective capacity of BCG. This study therefore defines the genetic basis of a compromise that has occurred, during the attenuation process, between loss of virulence and reduced protection (M. A. Behr, P.

M. Small, *Nature* **389**, 133 (1997)). The recombinant BCGs presented here may not be appropriate in their current form as vaccine candidates because of uncertainty about their safety. However, the strategy of reintroducing, or even overproducing (M. A. Horwitz et al, *Proc Natl Acad Sci U S A* **97**, 13853 (2000)), the missing immunodominant antigens of *M. tuberculosis* in BCG, could be combined with an immuno-neutral attenuating mutation to create a more efficacious tuberculosis vaccine.

Example 2: BAC based comparative genomics identifies *Mycobacterium microti* as a natural ESAT-6 deletion mutant.

We searched for any genetic differences between human and vole isolates that might explain their different degree of virulence and host preference and what makes the vole isolates harmless for humans. In this regard, comparative genomics methods were employed in connection with the present invention to identify major differences that may exist between the *M. microti* reference strain OV254 and the entirely sequenced strains of *M. tuberculosis* H37Rv (10) or *M. bovis* AF2122/97 (14). An ordered Bacterial Artificial Chromosome (BAC) library of *M. microti* OV254 was constructed and individual BAC to BAC comparison of a minimal set of these clones with BAC clones from previously constructed libraries of *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 was undertaken.

Ten regions were detected in *M. microti* that were different to the corresponding genomic regions in *M. tuberculosis* and *M. bovis*. To investigate if these regions were associated with the ability of *M. microti* strains to infect humans, their genetic organization was studied in 8 additional *M. microti* strains, including those isolated recently from patients with pulmonary tuberculosis. This analysis identified some regions that were specifically absent from all tested *M. microti* strains, but present in all

other members of the *M. tuberculosis* complex and other regions that were only absent from vole isolates of *M. microti*.

2.1 MATERIALS AND METHODS

Bacterial strains and plasmids. *M. microti* OV254 which was originally isolated from voles in the UK in the 1930's was kindly supplied by MJ Colston (45). DNA from *M. microti* OV216 and OV183 were included in a set of strains used during a multicenter study (26). *M. microti* Myc 94-2272 was isolated in 1988 from the perfusion fluid of a 41-year-old dialysis patient (43) and was kindly provided by L. M. Parsons. *M. microti* 35782 was purchased from American Type Culture Collection (designation TMC 1608 (M.P. Prague)). *M. microti* B1 type llama, B2 type llama, B3 type mouse and B4 type mouse were obtained from the collection of the National Reference Center for Mycobacteria, Forschungszentrum Borstel, Germany. *M. bovis* strain AF2122/97, spoligotype 9 was responsible for a herd outbreak in Devon in the UK and has been isolated from lesions in both cattle and badgers. Typically, mycobacteria were grown on 7H9 Middlebrook liquid medium (Difco) containing 10% oleic-acid-dextrose-catalase (Difco), 0.2 % pyruvic acid and 0.05% Tween 80.

Library construction, preparation of BAC DNA and sequencing reactions. Preparation of agarose-embedded genomic DNA from *M. microti* strain OV254, *M. tuberculosis* H37Rv, *M. bovis* BCG was performed as described by Brosch et al. (5). The *M. microti* library was constructed by ligation of partially digested *Hind*III fragments (50-125 kb) into pBeloBAC11. From the 10,000 clones that were obtained, 2,000 were picked into 96 well plates and stored at −80°C. Plasmid preparations of recombinant clones for sequencing reactions were obtained by pooling eight copies of 96 well plates, with each well containing an overnight culture in 250 µl 2YT medium with 12.5 $\mu g.ml^{-1}$

chloramphenicol. After 5 min centrifugation at 3000 rpm, the bacterial pellets were resuspended in 25 µl of solution A (25 mM Tris, pH 8.0, 50 mM glucose and 10 mM EDTA), cells were lysed by adding 25 µl of buffer B (NaOH 0.2 M, SDS 0.2%). Then 20 µl of cold 3 M sodium acetate pH 4.8 were added and kept on ice for 30 min. After centrifugation at 3000 rpm for 30 min, the pooled supernatants (140 µl) were transferred to new plates. 130 µl of isopropanol were added, and after 30 min on ice, DNA was pelleted by centrifugation at 3500 rpm for 15 min. The supernatant was discarded and the pellet resuspended in 50 µl of a 10 µg/ml RNAse A solution (in Tris 10 mM pH 7.5 / EDTA 10 mM) and incubated at 64°C for 15 min. After precipitation (2.5 µl of sodium acetate 3 M pH 7 and 200 µl of absolute ethanol) pellets were rinsed with 200 µl of 70% ethanol, air dried and finally suspended in 20 µl of TE buffer.

End-sequencing reactions were performed with a *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) using a mixture of 13 µl of DNA solution, 2 µl of Primer (2 µM) (SP6-BAC1, AGTTAGCTCACTCATTAGGCA (SEQ ID No 7), or T7-BAC1, GGATGTGCTGCAAGGCGATTA (SEQ ID No 8)), 2.5 µl of Big Dye and 2.5 µl of a 5X buffer (50 mM $MgCl_2$, 50 mM Tris). Thermal cycling was performed on a PTC-100 amplifier (MJ Inc.) with an initial denaturation step of 60 s at 95°C, followed by 90 cycles of 15 s at 95°C, 15 s at 56°c, 4 min at 60°C. DNA was then precipitated with 80 µl of 76% ethanol and centrifuged at 3000 rpm for 30 min. After discarding the supernatant, DNA was finally rinsed with 80 µl of 70% ethanol and resuspended in appropriate buffers depending on the type of automated sequencer used (ABI 377 or ABI 3700). Sequence data were transferred to Digital workstations and edited using the TED software from the Staden package (37). Edited sequences were compared against the *M. tuberculosis* H37Rv database (http://genolist.pasteur.fr/TubercuList/), the *M. bovis* BLAST server (http://www.sanger.ac.uk/Projects/M_bovis/blast_server.shtml), and in-house databases to determine the relative positions of the *M. microti* OV254 BAC end-sequences.

Preparation of BAC DNA from recombinants and BAC digestion profile comparison. DNA for digestion was prepared as previously described (4). DNA (1 µg) was digested with *Hind*III (Boehringer) and restriction products separated by pulsed-field gel electrophoresis (PFGE) on a Biorad CHEF-DR III system using a 1% (w/v) agarose gel and a pulse of 3.5 s for 17 h at 6 $V.cm^{-1}$. Low-range PFGE markers (NEB) were used as size standards. Insert sizes were estimated after ethidium bromide staining and visualization with UV light. Different comparisons were made with overlapping clones from the *M. microti* OV254, *M. bovis* AF2122/97, and *M. tuberculosis* H37Rv pBeloBAC11 libraries.

PCR analysis to determine presence of genes in different *M. microti* strains. Reactions contained 5 µl of 10xPCR buffer (100 mM ß-mercaptoethanol, 600 mM Tris-HCl, pH 8.8, 20 mM $MgCl_2$, 170 mM $(NH_4)_2SO_4$, 20 mM nucleotide mix dNTP), 2.5 µl of each primer at 2 µM, 10 ng of template DNA, 10% DMSO and 0.5 unit of Taq polymerase in a final volume of 12.5 µl. Thermal cycling was performed on a PTC-100 amplifier (MJ Inc.) with an initial denaturation step of 90 s at 95°C, followed by 35 cycles of 45 s at 95°C, 1 min at 60°C and 2 min at 72°C.

RFLP analysis. In brief, agarose plugs of genomic DNA prepared as previously described (5) were digested with either *Ase*I, *Dra*I or *Xba*I (NEB), then electrophoresed on a 1% agarose gel, and finally transferred to Hybond-C extra nitrocellulose membranes (Amersham). Different probes were amplified by PCR from the *M. microti* strain OV254 or *M. tuberculosis* H37Rv using primers for :

esat-6 (esat-6F GTCACGTCCATTCATTCCCT (SEQ ID No 9);
esat-6R ATCCCAGTGACGTTGCCTT) (SEQ ID No 10),
the $RD1^{mic}$ flanking region (4340, 209F GCAGTGCAAAGGTGCAGATA (SEQ ID No 11); 4354,701R GATTGAGACACTTGCCACGA (SEQ ID No 12)), or

plcA (plcA.int.F CAAGTTGGGTCTGGTCGAAT (SEQ ID No 13); plcA.int.R GCTACCCAAGGTCTCCTGGT (SEQ ID No 14)). Amplification products were radio-labeled by using the Stratagene Prime-It II kit (Stratagene). Hybridizations were performed at 65°C in a solution containing NaCl 0.8 M, EDTA pH 8, 5 mM, sodium phosphate 50 mM pH 8, 2% SDS, 1X Denhardt's reagent and 100 µg/ml salmon sperm DNA (Genaxis). Membranes were exposed to phosphorimager screens and images were digitalized by using a STORM phospho-imager.

DNA sequence accession numbers. The nucleotide sequences that flank MiD1, MiD2, MiD3 as well as the junction sequence of $RD1^{mic}$ have been deposited in the EMBL database. Accession numbers are AJ345005, AJ345006, AJ315556 and AJ315557, respectively.

2.2 RESULTS

Establishment of a complete ordered BAC library of *M. microti* OV254. Electroporation of pBeloBAC11 containing partial *Hind*III digests of *M. microti* OV254 DNA into *Escherichia coli* DH10B yielded about 10,000 recombinant clones, from which 2,000 were isolated and stored in 96-well plates. Using the complete sequence of the *M. tuberculosis* H37Rv genome as a scaffold, end-sequencing of 384 randomly chosen *M. microti* BAC clones allowed us to select enough clones to cover almost all of the 4.4 Mb chromosome. A few rare clones that spanned regions that were not covered by this approach were identified by PCR screening of pools as previously described (4). This resulted in a minimal set of 50 BACs, covering over 99.9% of the *M. microti* OV254 genome, whose positions relative to *M. tuberculosis* H37Rv are shown in Figure 5. The insert size ranged between 50 and 125 kb, and the recombinant clones were stable. Compared with other BAC libraries from tubercle bacilli (4, 13) the *M. microti* OV254 BAC library contained clones that were generally larger than those obtained previously, which facilitated the comparative genomics approach, described below.

Identification of DNA deletions in *M. microti* OV254 relative to *M. tuberculosis* H37Rv by comparative genomics. The minimal overlapping set of 50 BAC clones, together with the availability of three other ordered BAC libraries from *M. tuberculosis* H37Rv, *M. bovis* BCG Pasteur 1173P2 (5, 13) and *M. bovis* AF2122/97 (14) allowed us to carry out direct BAC to BAC comparison of clones spanning the same genomic regions. Size differences of PFGE-separated *Hind*III restriction fragments from *M. microti* OV254 BACs, relative to restriction fragments from *M. bovis* and/or *M. tuberculosis* BAC clones, identified loci that differed among the tested strains. Size variations of at least 2 kb were easily detectable and 10 deleted regions, evenly distributed around the genome, and containing more than 60 open reading frames (ORFs), were identified. These regions represent over 60 kb that are missing from *M. microti* OV254 strain compared to *M. tuberculosis* H37Rv. First, it was found that phiRv2 (RD11), one of the two *M. tuberculosis* H37Rv prophages was present in *M. microti* OV254, whereas phiRv1, also referred to as RD3 (29) was absent. Second, it was found that *M. microti* lacks four of the genomic regions that were also absent from *M. bovis* BCG. In fact, these four regions of difference named RD7, RD8, RD9 and RD10 are absent from all members of the *M. tuberculosis* complex with the exception of *M. tuberculosis* and *M. canettii*, and seem to have been lost from a common progenitor strain of *M. africanum*, *M. microti* and *M. bovis* (3). As such, our results obtained with individual BAC to BAC comparisons show that *M. microti* is part of this non-*M. tuberculosis* lineage of the tubercle bacilli, and this assumption was further confirmed by sequencing the junction regions of RD7 – RD10 in *M. microti* OV254. The sequences obtained were identical to those from *M. africanum*, *M. bovis* and *M. bovis* BCG strains. Apart from these four conserved regions of difference, and phiRv1 (RD3) *M. microti* OV254 did not show any other RDs with identical junction regions to *M. bovis* BCG Pasteur, which misses at least 17 RDs relative to *M. tuberculosis* H37Rv (1, 13, 35). However, five other regions missing from the genome of *M. microti* OV254 relative to

M. tuberculosis H37Rv were identified (RD1^{mic}, RD5^{mic}, MiD1, MiD2, MiD3). Such regions are specific either for strain OV254 or for *M. microti* strains in general. Interestingly, two of these regions, RD1^{mic}, RD5^{mic} partially overlap RDs from the *M. bovis* BCG.

Antigens ESAT-6 and CFP-10 are absent from *M. microti*. One of the most interesting findings of the BAC to BAC comparison was a novel deletion in a genomic region close to the origin of replication (figure 5). Detailed PCR and sequence analysis of this region in *M. microti* OV254 showed a segment of 14 kb to be missing (equivalent to *M. tuberculosis* H37Rv from 4340,4 to 4354,5 kb) that partly overlapped RD1^{bcg} absent from *M. bovis* BCG. More precisely, ORFs Rv3864 and Rv3876 are truncated in *M. microti* OV254 and ORFs Rv3865 to Rv3875 are absent (figure 6). This observation is particularly interesting as previous comparative genomic analysis identified RD1^{bcg} as the only RD region that is specifically absent from all BCG sub-strains but present in all other members of the *M. tuberculosis* complex (1, 4, 13, 29, 35). As shown in Figure 6, in *M. microti* OV254 the RD1^{mic} deletion is responsible for the loss of a large portion of the conserved ESAT-6 family core region (40) including the genes coding for the major T-cell antigens ESAT-6 and CFP-10 (2, 15). The fact that previous deletion screening protocols employed primer sequences that were designed for the right hand portion of the RD1^{bcg} region (i.e. gene Rv3878) (6, 39) explains why the RD1^{mic} deletion was not detected earlier by these investigations. Figure 6 shows that RD1^{mic} does not affect genes Rv3877, Rv3878 and Rv3879 which are part of the RD1 ^{bcg} deletion.

Deletion of phospholipase-C genes in *M. microti* OV254. RD5^{mic}, the other region absent from *M. microti* OV254, that partially overlapped an RD region from BCG, was revealed by comparison of BAC clone Mi18A5 with BAC Rv143 (figure 5). PCR analysis and sequencing of the junction region revealed that RD5^{mic} was smaller than the RD5 deletion in BCG (Table 1 and 2 below).

TABLE 1.

Description of the putative function of the deleted and truncated ORFs in *M. microti* OV254

Region	Start - End	overlapping ORF	Putative Function or family
RD 10	264,5-266,5	Rv0221-Rv0223	*echA1*
RD 3	1779,5-1788,5	Rv1573-Rv1586	bacteriophage proteins
RD 7	2207,5-2220,5	Rv1964-Rv1977	*yrbE3A-3B; mce3A-F;* unknown
RD 9	2330-2332	Rv2072-Rv2075	*cobL;* probable oxidoreductase; unknown
RD5^{mic}	2627,6-2633,4	Rv2348-Rv2352	*plcA-C;* member of PPE family
MiD1	3121,8-3126,6	Rv2816-Rv2819	IS*6110* transposase; unknown
MiD2	3554,0-3755,2	Rv3187-Rv3190	IS*6110* transposase; unknown
MiD3	3741,1-3755,7	Rv3345-Rv3349	members of the PE-PGRS and PPE families; insertion elements
RD8	4056,8-4062,7	Rv3617-Rv3618	*ephA*; *lpqG;* member of the PE-PGRS family
RD1^{mic}	4340,4-4354,5	Rv3864-Rv3876	member of the CBXX/CF QX family; member of the PE and PPE families; ESAT-6; CFP10; unknown

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TABLE 2. Sequence at the junction of the deleted regions in *M. microti* OV254

Junction	Position	ORFs	Sequences at the junction	Flanking primers
RD1^{mic} (SEQ ID No 15)	4340,421-4354,533	Rv3864-Rv3876	**CAAGACGAGGTTGTAAAACCTCGACG** **CAGGATCGGCGATGAAATGCCAGTCG** **GCGTCGCTGAGCGCGCGCTGCGC***CGA* *GTCCCATTTTGTCGCTGATTTGTTTGAACA* *GCGACGAACCGGTGTTGAAAATGTCGCCT* *GGGTCGGGGATTCCCT*	4340,209F (SEQ ID No 11) GCAGTGCAAAGGTGCAGATA 4354,701R (SEQ ID No 12) GATTGAGACACTTGCCACGA
RD5^{mic} (SEQ ID No 18)	2627,831-2635,581	Rv2349-Rv2355	**CCTCGATGAACCACCTGACATGACCC** **CATCCTTTCCAAGAACTGGAGTCTCC** **GGACATGCCGGGGCGGTTCA***CTGCCC* *CAGGTGTCCTGGGTCGTTCCGTTGACCGT*	2627,370F (SEQ ID No 16) GAATGCCGACGTCATATCG 2633,692R (SEQ ID No 17)

			CGAGTCCGAACATCCGTCATTCCCGGTGG CAGTCGGTGCGGTGAC	CGGCCACTGAGTTCGATTAT
MiD1 (SEQ ID No 21)	3121,880-3126,684	Rv2815c-Rv2818c	CACCTGACATGACCCCATCCTTTCCA AGAACTGGAGTCTCCGGACATGCCGG GGCGGTTCAG*GGACATTCATGTCCATCTT CTGGCAGATCAGCAGATCGCTTGTTCTCAG TGCAGGTGAGTC*	3121,690F (SEQ ID No 19) CAGCCAACACCAAGTAGACG 3126,924R (SEQ ID No 20) TCTACCTGCAGTCGCTTGTG
MiD2 (SEQ ID No 24)	3554,066-3555,259	Rv3188-Rv3189	GCTGCCTACTACGCTCAACGCCAGAG ACCAGCCGCCGGCTGAGGTCTCAGAT CAGAGAGTCTCCGGACTCACCGGGGC GGTTCA*TAAAGGCTTCGAGACCGGACGG GCTGTAGGTTCCTCAACTGTGTGGCGGAT GGTCTGAGCACTTAAC*	3553,880F (SEQ ID No 22) GTCCATCGAGGATGTCGAGT 3555,385R (SEQ ID No 23) CTAGGCCATTCCGTTGTCTG
MiD3 (SEQ ID No 27)	3741,139-3755,777	Rv3345c-Rv3349c	TGGCGCCGGCACCTCCGTTGCCACCG TTGCCGCCGCTGGTGGGCGCGGTGCC GTTCGCCCCGGCCGAACCGTTCAGGG CCGGGTTC*GCCCTCAGCCGCTAAACACG CCGACCAAGATCAACGAGCTACCTGCCCG GTCAAGGTTGAAGAGCCCCCATATCAGCA AGGGCCCGGTGTCGGCG*	3740,950F (SEQ ID No 25) GGCGACGCCATTTCC 3755,988R (SEQ ID No 26) AACTGTCGGGCTTGCTCTT

In fact, *M. microti* OV254 lacks the genes *plcA, plcB, plcC* and two specific PPE-protein encoding genes (Rv2352, Rv2353). This was confirmed by the absence of a clear band on a Southern blot of *Ase*I digested genomic DNA from *M. microti* OV254 hybridized with a *plcA* probe. However, the genes Rv2346c and Rv2347c, members of the *esat-6* family, and Rv2348c, that are missing from *M. bovis* and BCG strains (3) are still present in *M. microti* OV254. The presence of an IS*6110* element in this segment suggests that recombination between two IS*6110* elements could have been involved in the loss of RD5^{mic}, and this is supported by the finding that the remaining copy of IS*6110* does not show a 3 base-pair direct repeat in strain OV254 (Table 2).

Lack of MiD1 provides genomic clue for *M. microti* OV254 characteristic spoligotype. MiD1 encompasses the three ORFs Rv2816, Rv2817 and Rv2818 that encode putative proteins whose functions are yet unknown, and has occurred in the direct repeat region (DR), a polymorphic locus in the genomes of the tubercle bacilli that contains a cluster of direct repeats of 36 bp, separated by unique spacer sequences of 36 to 41 bp (17), (figure 7). The presence or absence of 43 unique spacer sequences that

intercalate the DR sequences is the basis of spacer-oligo typing, a powerful typing method for strains from the *M. tuberculosis* complex (23). *M. microti* isolates exhibit a characteristic spoligotype with an unusually small DR cluster, due to the presence of only spacers 37 and 38 (43). In *M. microti* OV254, the absence of spacers 1 to 36, which are present in many other *M. tuberculosis* complex strains, appears to result from an IS*6110* mediated deletion of 636 bp of the DR region. Amplification and *Pvu*II restriction analysis of a 2.8 kb fragment obtained with primers located in the genes that flank the DR region (Rv2813c and Rv2819) showed that there is only one copy of IS*6110* remaining in this region (figure 7). This IS*6110* element is inserted into ORF Rv2819 at position 3,119,932 relative to the *M. tuberculosis* H37Rv genome. As for other IS*6110* elements that result from homologous recombination between two copies (7), no 3 base-pair direct repeat was found for this copy of IS*6110* in the DR region. Concerning the absence of spacers 39-43 (figure7), it was found that *M. microti* showed a slightly different organization of this locus than *M. bovis* strains, which also characteristically lack spacers 39-43. In *M. microti* OV254 an extra spacer of 36 bp was found that was not present in *M. bovis* nor in *M. tuberculosis* H37Rv. The sequence of this specific spacer was identical to that of spacer 58 reported by van Embden and colleagues (42). In their study of the DR region in many strains from the *M. tuberculosis* complex this spacer was only found in *M. microti* strain NLA000016240 (AF189828) and in some ancestral *M. tuberculosis* strains (3, 42). Like MiD1, MiD2 most probably results from an IS*6110*-mediated deletion of two genes (Rv3188, Rv3189) that encode putative proteins whose function is unknown (Table 2 above and Table 3 below).

TABLE 3. Presence of the RD and MiD regions in different *M. microti* strains

HOST		VOLES					HUMAN		
Strain	*OV254*	OV183	*OV216*	ATCC 35782	*Myc 94 -2272*	B3 type mouse	B4 type mouse	B1 type llama	B2 type llama

RD1^{mic}	absent	absent	absent	absent	absent	absent	absent	absent	absent
RD 3	absent	absent	absent	absent	absent	absent	absent	absent	absent
RD 7	absent	absent	absent	absent	absent	absent	absent	absent	absent
RD8	absent	absent	absent	absent	absent	absent	absent	absent	absent
RD 9	absent	absent	absent	absent	absent	absent	absent	absent	absent
RD 10	absent	absent	absent	absent	absent	absent	absent	absent	absent
MiD3	absent	ND	ND	absent	absent	absent	absent	absent	absent
MiD1	absent	ND	ND	present	partial	partial	partial	present	present
RD5^{mic}	absent	absent	absent	present	present	present	present	present	present
MiD2	absent	ND	ND	present	present	present	present	present	present

ND, not determined

Absence of some members of the PPE family in *M. microti*. MiD3 was identified by the absence of two *Hind*III sites in BAC Mi4B9 that exist at positions 3749 kb and 3754 kb in the *M. tuberculosis* H37Rv chromosome. By PCR and sequence analysis, it was determined that MiD3 corresponds to a 12 kb deletion that has truncated or removed five genes orthologous to Rv3345c-Rv3349c. Rv3347c encodes a protein of 3157 amino-acids that belongs to the PPE family and Rv3346c a conserved protein that is also present in *M. leprae*. The function of both these putative proteins is unknown while Rv3348 and Rv3349 are part of an insertion element (Table 1). At present, the consequences of the MiD3 deletions for the biology of *M. microti* remains entirely unknown.

Extra-DNA in *M. microti* OV254 relative to *M. tuberculosis* H37Rv. *M. microti* OV254 possesses the 6 regions RvD1 to RvD5 and TBD1 that are absent from the sequenced strain *M. tuberculosis* H37Rv, but which have been shown to be present in other members of the *M. tuberculosis* complex, like *M. canettii*, *M. africanum*, *M. bovis*,

and *M. bovis* BCG (3, 7, 13). In *M. tuberculosis* H37Rv, four of these regions (RvD2-5) contain a copy of IS*6110* which is not flanked by a direct repeat, suggesting that recombination of two IS*6110* elements was involved in the deletion of the intervening genomic regions (7). In consequence, it seems plausible that these regions were deleted from the *M. tuberculosis* H37Rv genome rather than specifically acquired by *M. microti*. In addition, three other small insertions have also been found and they are due to the presence of an IS*6110* element in a different location than in *M. tuberculosis* H37Rv and *M. bovis* AF2122/97. Indeed, *Pvu*II RFLP analysis of *M. microti* OV254 reveals 13 IS*6110* elements (data not shown).

Genomic diversity of *M. microti* strains. In order to obtain a more global picture of the genetic organization of the taxon *M. microti* we evaluated the presence or absence of the variable regions found in strain OV254 in eight other *M. microti* strains. These strains which were isolated from humans and voles have been designated as *M. microti* mainly on the basis of their specific spoligotype (26, 32, 43) and can be further divided into subgroups according to the host such as voles, llama and humans (Table 3). As stated in the introduction, *M. microti* is rarely found in humans unlike *M. tuberculosis*. So the availability of 9 strains from variable sources for genetic characterization is an exceptional resource. Among them was one strain (Myc 94-2272) from a severely immuno-compromised individual (43), and four strains were isolated from HIV-positive or HIV-negative humans with spoligotypes typical of llama and mouse isolates. For one strain, ATCC 35872 / M.P. Prague, we could not identify with certainty the original host from which the strain was isolated, nor if this strain corresponds to *M. microti* OV166, that was received by Dr. Sula from Dr. Wells and used thereafter for the vaccination program in Prague in the 1960's (38).

First, we were interested if these nine strains designated as *M. microti* on the basis of their spoligotypes also resembled each other by other molecular typing criteria. As RFLP

of pulsed-field gel separated chromosomal DNA represents probably the most accurate molecular typing strategy for bacterial isolates, we determined the *Ase*I profiles of the available *M. microti* strains, and found that the profiles resembled each other closely but differed significantly from the macro-restriction patterns of *M. tuberculosis*, *M. bovis* and *M. bovis* BCG strains used as controls. However, as depicted in Figure 8A, the patterns were not identical to each other and each *M. microti* strain showed subtle differences, suggesting that they were not epidemiologically related. A similar observation was made with other rare cutting restriction enzymes, like *Dra*I or *Xba*I (data not shown).

Common and diverging features of *M. microti* strains. Two strategies were used to test for the presence or absence of variable regions in these strains for which we do not have ordered BAC libraries. First, PCRs using internal and flanking primers of the variable regions were employed and amplification products of the junction regions were sequenced. Second, probes from the internal portion of variable regions absent from *M. microti* OV254 were obtained by amplification of *M. tuberculosis* H37Rv DNA using specific primers. Hybridization with these radio-labeled probes was carried out on blots from PFGE separated *Ase*I restriction digests of the *M. microti* strains. In addition, we confirmed the findings obtained by these two techniques by using a focused macro-array, containing some of the genes identified in variable regions of the tubercle bacilli to date (data not shown).

This led to the finding that the RD1^{mic} deletion is specific for all *M. microti* strains tested.

Indeed, none of the *M. microti* DNA-digests hybridized with the radio-labeled *esat-6* probe (Fig. 8B) but with the RD1^{mic} flanking region (Fig. 8C). In addition, PCR amplification using primers flanking the RD1^{mic} region (Table 2) yielded fragments of the same size for *M. microti* strains whereas no products were obtained for *M.*

tuberculosis, *M. bovis* and *M. bovis* BCG strains (Fig. 9). Furthermore, the sequence of the junction region was found identical among the strains which confirms that the genomic organization of the RD1^{mic} locus was the same in all tested *M. microti* strains (Table 3). This clearly demonstrates that *M. microti* lacks the conserved ESAT-6 family core region stretching in other members of the *M. tuberculosis* complex from Rv3864 to Rv3876 and, as such, represents a taxon of naturally occurring ESAT-6 / CFP-10 deletion mutants.

Like RD1^{mic}, MiD3 was found to be absent from all nine *M. microti* strains tested and, therefore, appears to be a specific genetic marker that is restricted to *M. microti* strains (Table 3). However, PCR amplification showed that RD5^{mic} is absent only from the vole isolates OV254, OV216 and OV183, but present in the *M. microti* strains isolated from human and other origins (Table 3). This was confirmed by the presence of single bands but of differing sizes on a Southern blot hybridized with a *plcA* probe for all *M. microti* tested strains except OV254 (Fig. 8D). Interestingly, the presence or absence of RD5^{mic} correlated with the similarity of IS*6110* RFLP profiles. The profiles of the three *M. microti* strains isolated from voles in the UK differed considerably from the IS*6110* RFLP patterns of humans isolates (43). Taken together, these results underline the proposed involvement of IS*6110* mediated deletion of the RD5 region and further suggest that RD5 may be involved in the variable potential of *M. microti* strains to cause disease in humans. Similarly, it was found that MiD1 was missing only from the vole isolates OV254, OV216 and OV183, which display the same spoligotype (43), confirming the observations that MiD1 confers the particular spoligotype of a group of *M. microti* strains isolated from voles. In contrast, PCR analysis revealed that MiD1 is only partially deleted from strains B3 and B4 both characterized by the mouse spoligotype and the human isolate *M. microti* Myc 94-2272 (Table 3). For strain ATCC 35782 deletion of the MiD1 region was not observed. These findings correlate with the described spoligotypes of the different isolates, as strains that had intact or partially

deleted MiD1 regions had more spacers present than the vole isolates that only showed spacers 37 and 38.

2.3 COMMENTS AND DISCUSSION

We have searched for major genomic variations, due to insertion-deletion events, between the vole pathogen, *M. microti,* and the human pathogen, *M. tuberculosis*. BAC based comparative genomics led to the identification of 10 regions absent from the genome of the vole bacillus *M. microti* OV254 and several insertions due to IS*6110*. Seven of these deletion regions were also absent from eight other *M. microti* strains, isolated from voles or humans, and they account for more than 60 kb of genomic DNA. Of these regions, $RD1^{mic}$ is of particular interest, because absence of part of this region has been found to be restricted to the BCG vaccine strains to date. As *M. microti* was originally described as non pathogenic for humans, it is proposed here that RD1 genes is involved in the pathogenicity for humans. This is reinforced by the fact that $RD1^{bcg}$ (29) has lost putative ORFs belonging to the *esat-6* gene cluster including the genes encoding ESAT-6 and CFP-10 (Fig. 6) (40). Both polypeptides have been shown to act as potent stimulators of the immune system and are antigens recognized during the early stages of infection (8, 12, 20, 34). Moreover, the biological importance of this RD1 region for mycobacteria is underlined by the fact that it is also conserved in *M. leprae*, where genes ML0047-ML0056 show high similarities in their sequence and operon organization to the genes in the *esat-6* core region of the tubercle bacilli (11). In spite of the radical gene decay observed in *M. leprae* the *esat-6* operon apparently has kept its functionality in this organism.

However, the RD1 deletion may not be the only reason why the vole bacillus is attenuated for humans. Indeed, it remains unclear why certain *M. microti* strains included in the present study that show exactly the same $RD1^{mic}$ deletion as vole isolates, have

been found as causative agents of human tuberculosis. As human *M. microti* cases are extremely rare, the most plausible explanation for this phenomenon would be that the infected people were particularly susceptible for mycobacterial infections in general. This could have been due to an immunodeficiency (32, 43) or to a rare genetic host predisposition such as interferon gamma- or IL-12 receptor modification (22).

In addition, the finding that human *M. microti* isolates differed from vole isolates by the presence of region RD5^{mic} may also have an impact on the increased potential of human *M. microti* isolates to cause disease. Intriguingly, BCG and the vole bacillus lack overlapping portions of this chromosomal region that encompasses three (*plcA*, *plcB*, *plcC*) of the four genes encoding phospholipase C (PLC) in *M. tuberculosis*. PLC has been recognized as an important virulence factor in numerous bacteria, including *Clostridium perfringens*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*, where it plays a role in cell to cell spread of bacteria, intracellular survival, and cytolysis (36, 41). To date, the exact role of PLC for the tubercle bacilli remains unclear. *plcA* encodes the antigen mtp40 which has previously been shown to be absent from seven tested vole and hyrax isolates (28). Phospholipase C activity in *M. tuberculosis*, *M. microti* and *M. bovis*, but not in *M. bovis* BCG, has been reported (21, 47). However, PLC and sphingomyelinase activities have been found associated with the most virulent mycobacterial species (21). The levels of phospholipase C activity detected in *M. bovis* were much lower than those seen in *M. tuberculosis* consistent with the loss of *plcABC*. It is likely, that *plcD* is responsible for the residual phospholipase C activity in strains lacking RD5, such as *M. bovis* and *M. microti* OV254. Indeed, the *plcD* gene is located in region RvD2 which is present in some but not all tubercle bacilli (13, 18). Phospholipase encoding genes have been recognized as hotspots for integration of IS*6110* and it appears that the regions RD5 and RvD2 undergo independent deletion processes more frequently than any other genomic regions (44). Thus, the virulence of

some *M. microti* strains may be due to a combination of functional phospholipase C encoding genes (7, 25, 26, 29).

Another intriguing detail revealed by this study is that among the deleted genes seven code for members of the PPE family of Gly-, Ala-, Asn-rich proteins. A closer look at the sequences of these genes showed that in some cases they were small proteins with unique sequences, like for example Rv3873, located in the RD1^{mic} region, or Rv2352c and Rv2353c located in the RD5^{mic} region. Others, like Rv3347c, located in the MiD3 region code for a much larger PPE protein (3157 aa). In this case a neighboring gene (Rv3345c), belonging to another multigene family, the PE-PGRS family, was partly affected by the MiD3 deletion. While the function of the PE/PPE proteins is currently unknown, their predicted abundance in the proteome of *M. tuberculosis* suggests that they may play an important role in the life cycle of the tubercle bacilli. Indeed, recently some of them were shown to be involved in the pathogenicity of *M. tuberculosis* strains (9). Complementation of such genomic regions in *M. microti* OV254 should enable us to carry out proteomics and virulence studies in animals in order to understand the role of such ORFs in pathogenesis.

In conclusion, this study has shown that *M. microti*, a taxon originally named after its major host *Microtus agrestis*, the common vole, represents a relatively homogenous group of tubercle bacilli. Although all tested strains showed unique PFGE macro-restriction patterns that differed slightly among each other, deletions that were common to all *M. microti* isolates (**RD7-RD10, MiD3, RD1^{mic}**) have been identified. The conserved nature of these deletions suggests that these strains are derived from a common precursor that has lost these regions, and their loss may account for some of the observed common phenotypic properties of *M. microti*, like the very slow growth on solid media and the formation of tiny colonies. This finding is consistent with results

from a recent study that showed that *M. microti* strains carry a particular mutation in the *gyrB* gene (31).

Of particular interest, some of these common features (e.g. the flanking regions of RD1^{mic}, or MiD3) could be exploited for an easy-to-perform PCR identification test, similar to the one proposed for a range of tubercle bacilli (33). This test enables unambiguous and rapid identification of *M. microti* isolates in order to obtain a better estimate of the overall rate of *M. microti* infections in humans and other mammalian species.

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CLAIMS

1. A strain of *M. bovis* BCG or *M. microti*, wherein said strain has integrated all or part of the RD1 region responsible for enhanced immunogenicity and increased persistence of BCG to the tubercle bacilli.

2. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis*, which comprises at least one gene selected from Rv3872 (SEQ ID No 2, mycobacterial PE), Rv3873 (SEQ ID No 3, PPE), Rv3874 (SEQ ID No 4, CFP-10), and Rv3875 (SEQ ID No 5, ESAT-6).

3. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis*, which comprises Rv3875 (SEQ ID No 5, ESAT-6).

4. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis*, which comprises Rv3874 (SEQ ID No 4, CFP-10).

5. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis*, which comprises both Rv3875 (SEQ ID No 5, ESAT-6) and (SEQ ID No 4, CFP-10).

6. A strain according to one of claims 2 to 5, wherein the coding sequence of the integrated gene is in frame with its natural promoter or with an exogenous promoter, such as a promoter capable of directing high level of expression of said coding sequence.

7. A strain according to one of claims 1 to 5, wherein said the integrated gene is mutated so as to maintain the improved immunogenicity while decreasing the virulence of the strain.

8. A strain according to claim 7, wherein said strain only carries parts of the genes coding for ESAT-6 or CFP-10 in a mycobacterial expression vector under the control of a promoter, more particularly an hsp60 promoter.

9. A strain according to claim 8, wherein said strain carries at least one portion of the esat-6 gene that codes for immunogenic 20-mer peptides of ESAT-6 active as T-cell epitopes.

10. A strain according to claim 7, wherein the esat-6 and CFP-10 encoding genes are altered by directed mutagenesis in a way that most of the immunogenic peptides of ESAT-6 remain intact, but the biological functionality of ESAT-6 is lost.

11. *M. bovis* BCG::RD1 strains which have integrated a cosmid herein referred as RD1-2F9 and RD1-AP34 contained in the E. coli strains deposited at the CNCM under the accession number I-2831 and I-2832 respectively.

12. *M. bovis* BCG::RD1 strain which has integrated the construct RD1-AP34 which contains a 3909 bp fragment of the M. tuberculosis H37Rv genome from region 4350459 bp to 4354367 bp cloned (SEQ ID No 1).

13. *M. bovis* BCG::RD1 strain which has integrated the fragment RD1-2F9 (~ 32 kb) that covers the region of the *M. tuberculosis* genome AL123456 from ca 4337 kb to ca. 4369 kb.

14. *M. microti*::RD1 strain which has integrated the construct RD1-AP34 which contains a 3909 bp fragment of the M. tuberculosis H37Rv genome from region 4350459 bp to 4354367 bp cloned (SEQ ID No 1).

15. *M. microti*::RD1 strain which has integrated the fragment RD1-2F9 (~ 32 kb) that covers the region of the *M. tuberculosis* genome AL123456 from ca 4337 kb to ca. 4369 kb.

16. A method for preparing and selecting improved *M. bovis* BCG or *M. microti* strains defined in one of claims 1 to 15 comprising a step consisting of modifying said strains by insertion, deletion or mutation in the integrated DR1 region, more particularly in the esat-6 or CFP-10 gene, said method leading to strains that are less virulent for immuno-depressed individuals.

17. A cosmid or a plasmid comprising all or part of the RD1 region originating from *Mycobacterium tuberculosis*, said region comprising at least one gene selected from Rv3872 (mycobacterial PE), Rv3873 (PPE), Rv3874 (CFP-10), and Rv3875 (ESAT-6).

18. A cosmid or a plasmid according to claim 17 comprising CFP-10, ESAT-6 or both or a part of them.

19. A cosmid or a plasmid according to claim 18 comprising a mutated gene selected CFP-10, ESAT-6 or both., said mutated gene being responsible for the improved immunogenicity and decreased virulence.

20. Use of a cosmid or a plasmid according to one of claims 17 to 19 for transforming *M. bovis* BCG or *M. microti.*

21. A pharmaceutical composition comprising a strain according to one of claims 1 to 15 and a pharmaceutically acceptable carrier.

22. A pharmaceutical composition according to claim 21 containing suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the living vaccine into preparations which can be used pharmaceutically.

23. A pharmaceutical composition according to claim 21 or 22 which is suitable for intravenous or subcutaneous administration.

24. A vaccine comprising a strain according to one of claims 1 to 15 and a suitable carrier.

25. A product comprising a strain according to one of claims 1 to 15 and at least one protein selected from ESAT-6 and CFP-10 or epitope derived thereof for a separate, simultaneous or sequential use for treating tuberculosis.

26. The use of a strain according to one of claims 1 to 15 for preparing a medicament or a vaccine for preventing or treating tuberculosis.

27. The use of a strain according to one of claims 1 to 15 as an adjuvant/immunomodulator for preparing a medicament for the treatment of superficial bladder cancer.

28. A method for the identification at the species level of members of the *M. tuberculosis* complex by means of markers for $RD1^{mic}$ and $RD5^{mic}$ as molecular diagnostic test.

29. A method according to claim 28 comprising the use of a primer selected from :

primer esat-6F GTCACGTCCATTCATTCCCT (SEQ ID No 9),
primer esat-6R ATCCCAGTGACGTTGCCTT) (SEQ ID No 10),
primer RD1^{mic} flanking region F GCAGTGCAAAGGTGCAGATA (SEQ ID No 11),
primer RD1^{mic} flanking region R GATTGAGACACTTGCCACGA (SEQ ID No 12),
primer RD5^{mic} flanking region F GAATGCCGACGTCATATCG (SEQ ID No 16),
primer RD5^{mic} flanking region R CGGCCACTGAGTTCGATTAT (SEQ ID No 17)
and the complementary sequences of said primers.

30. A diagnostic kit for the identification at the species level of members of the *M. tuberculosis* comprising DNA probes and primers specifically hybridizing to a DNA portion of the RD1 or RD5 region of M. tuberculosis, more particularly probes hybridizing under stringent conditions to a gene selected from Rv3871, Rv3872 (mycobacterial PE), Rv3873 (PPE), Rv3874 (CFP-10), Rv3875 (ESAT-6), and Rv3876, preferably CFP-10 and ESAT-6.

31. A diagnostic kit according to claim 30 comprising a probe or primer selected from :
esat-6F GTCACGTCCATTCATTCCCT (SEQ ID No 9),
esat-6R ATCCCAGTGACGTTGCCTT) (SEQ ID No 10),
RD1^{mic} flanking region F GCAGTGCAAAGGTGCAGATA (SEQ ID No 11),
RD1^{mic} flanking region R GATTGAGACACTTGCCACGA (SEQ ID No 12),
RD5^{mic} flanking region F GAATGCCGACGTCATATCG (SEQ ID No 16),
RD5^{mic} flanking region R CGGCCACTGAGTTCGATTAT (SEQ ID No 17).

32. A diagnostic kit for the identification at the species level of members of the *M. tuberculosis* comprising antibodies directed to mycobacterial PE, PPE, CFP-10 and ESAT-6.

33. Virulence markers associated with RD1 and/or RD5 regions of the genome of *M. tuberculosis* or a part of these regions.

ABSTRACT

The present invention relates to a strain of *M. bovis* BCG or *M. microti*, wherein said strain has integrated part or all of the RD1 region responsible for enhanced immunogenicity of the tubercle bacilli, especially the ESAT-6 and CFP-10 genes. These strains will be referred as the *M. bovis* BCG::RD1 or *M. microti*::RD1 strains and are useful as a new improved vaccine for preventing tuberculosis and as a therapeutical product enhancing the stimulation of the immune system for the treatment of bladder cancer.

FIGURE 1A

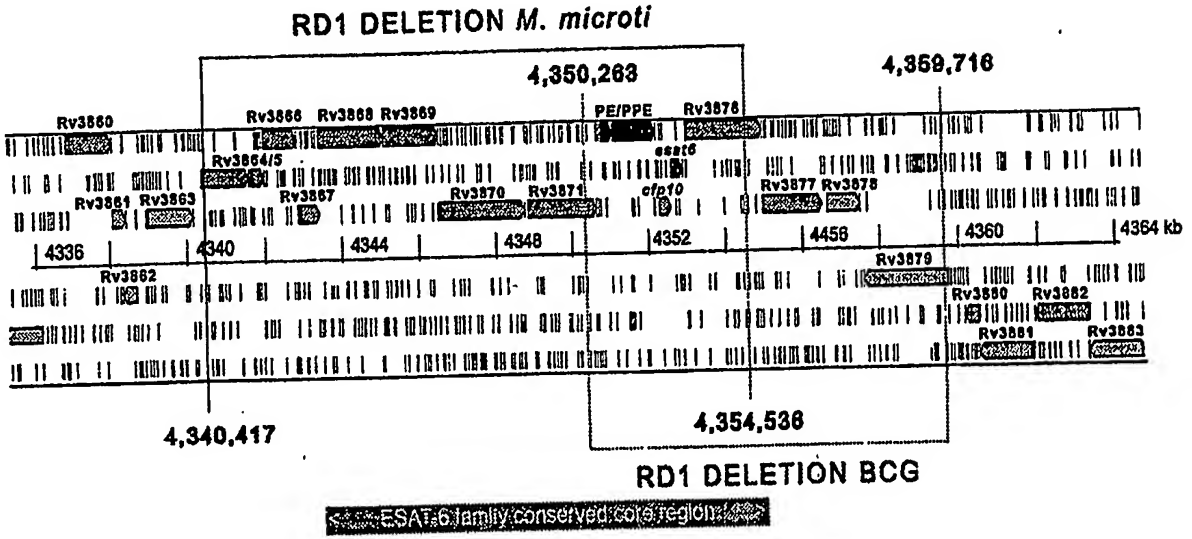


FIGURE 1B

Deleted Region	Coordinates (kilobase)	Putative virulence genes and their function	Integrating clones
RD3	1779-1788	Prophage phiRv1 (Rv1573-1586)	RD3-I301
RD4	1696-1708	Extracellular polysaccharide synthesis (Rv1511-Rv1514)	RD4-I375
RD5	2626-2635	Phospholipase operon (*plcA, plcB, plcC*)	RD5-1B1
RD7	2208-2220	Adhesin/invasin (*mce3* operon)	RD7-1B9
RD9	2330-2332	Cobalamin synthesis (*cobL*)	RD9-I493

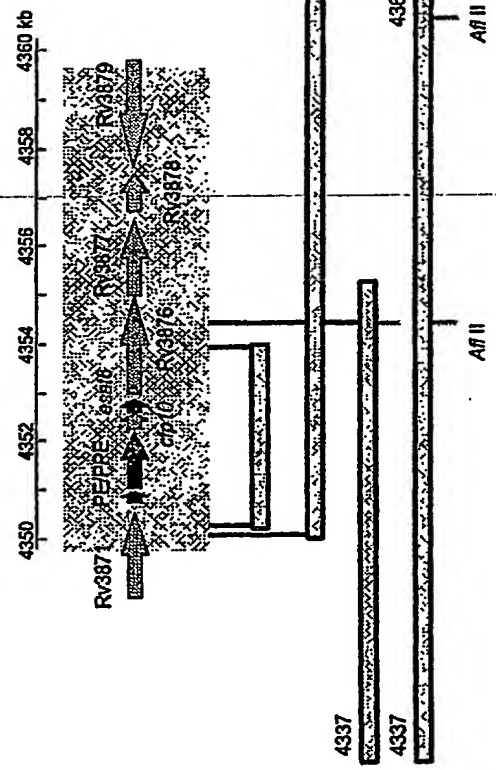


FIGURE 1C

FIGURE 1D

FIGURE 2A

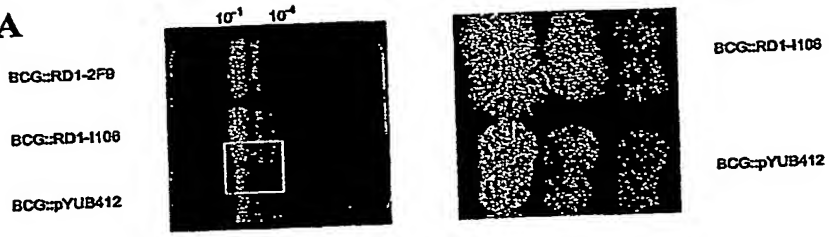


FIGURE 2B

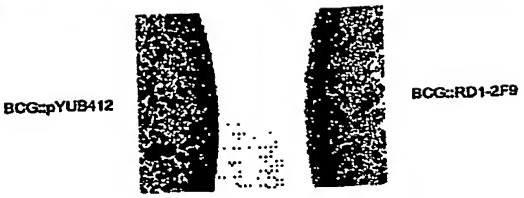


FIGURE 2C

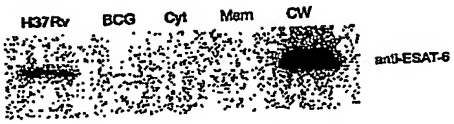


FIGURE 2D

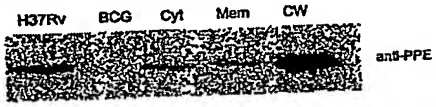


FIGURE 3A

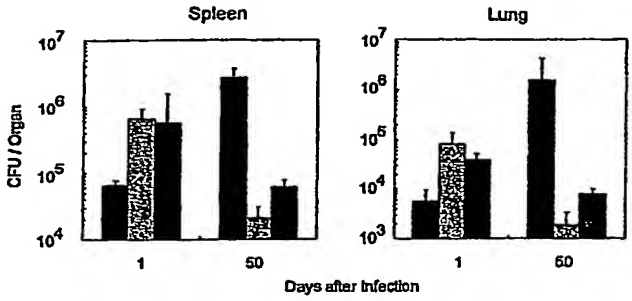


FIGURE 3B

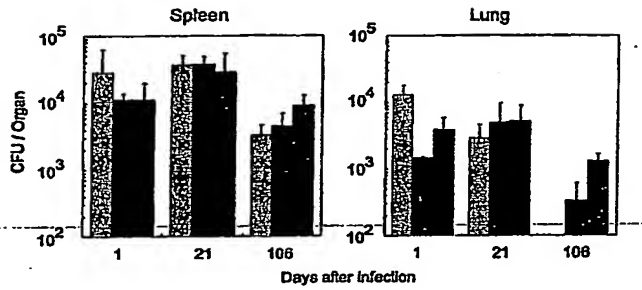


FIGURE 3C

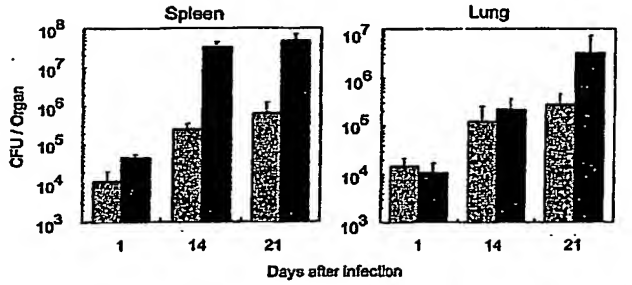


FIGURE 3D

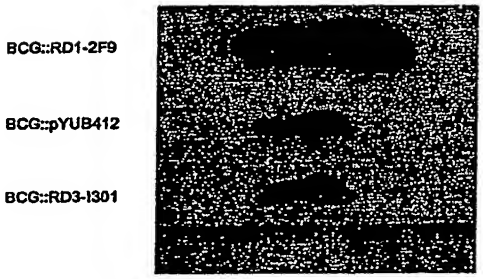


FIGURE 4A

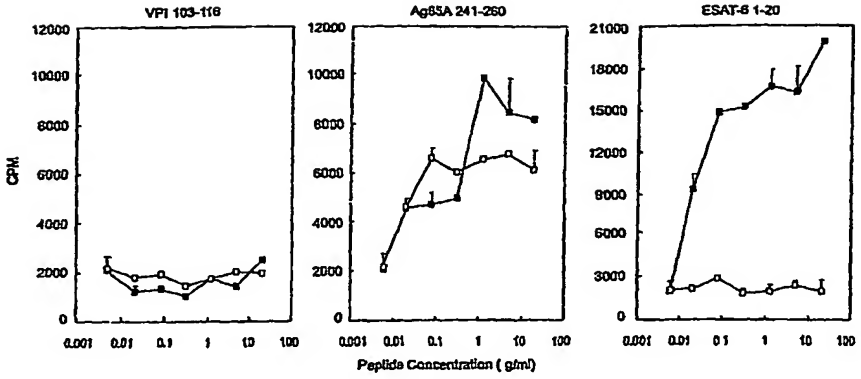


FIGURE 4B

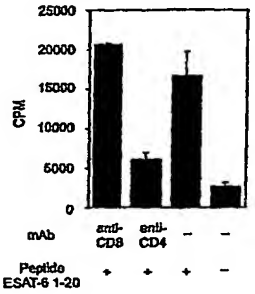


FIGURE 4C

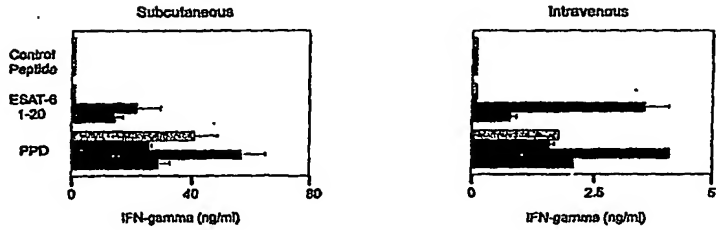
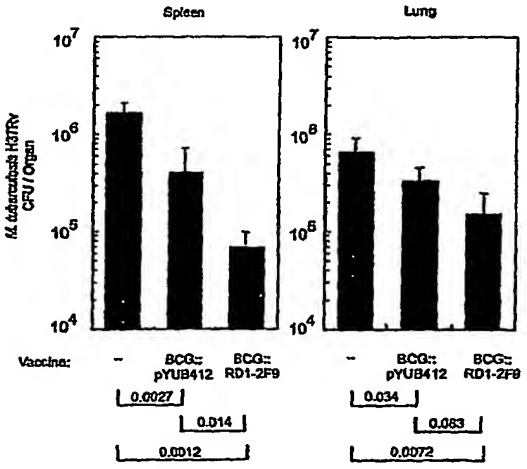


FIGURE 4D



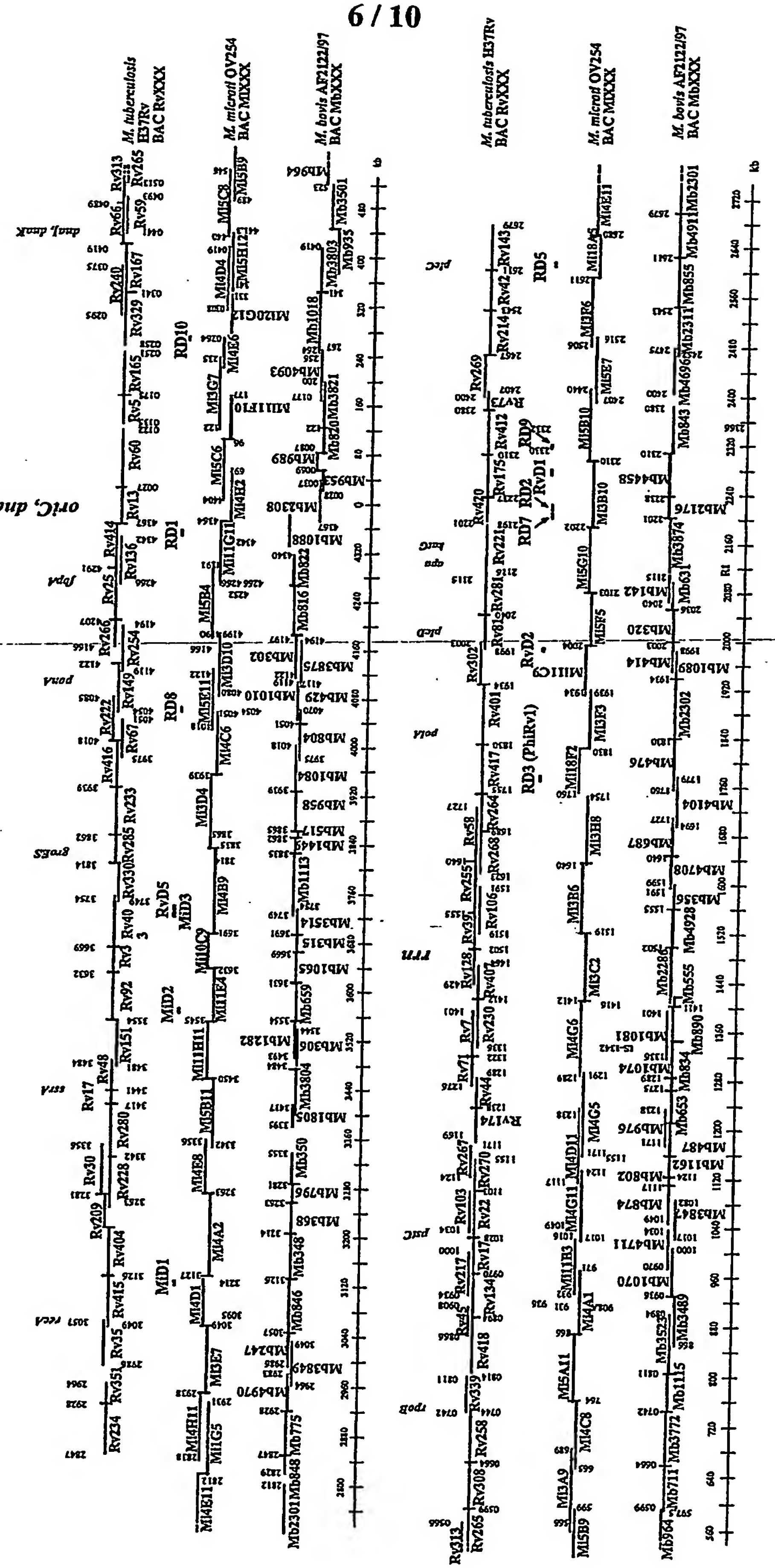

M. tuberculosis H37Rv BAC RvXXX
M. microti OV254 BAC MiXXX
M. bovis AF2122/97 BAC MbXXX
oriC, dnaA
RD1
RD2
RD3 (PhiRv1)
RD5
RD7
RD8
RD9
RD10
RvD1
RvD2
RvD5
MiD1
MiD2
MiD3
kb

FIGURE 5

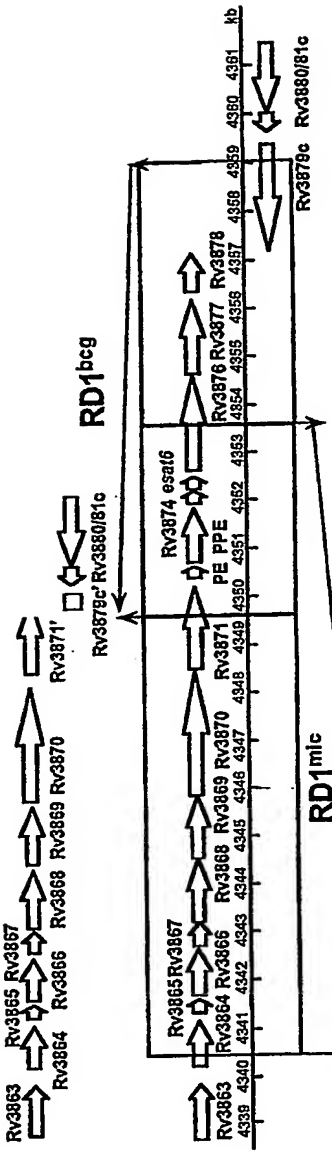


FIGURE 6A

FIGURE 6B

FIGURE 6C

FIGURE 7A

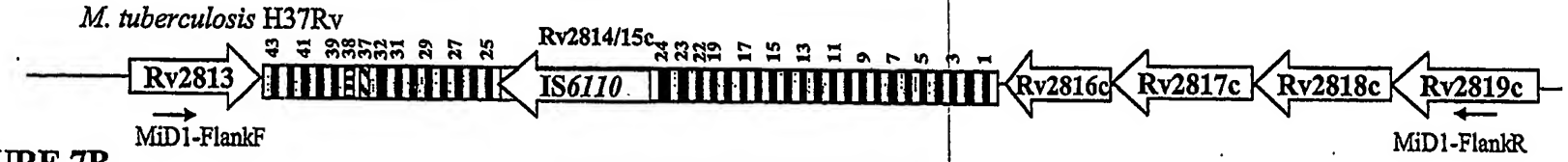


FIGURE 7B

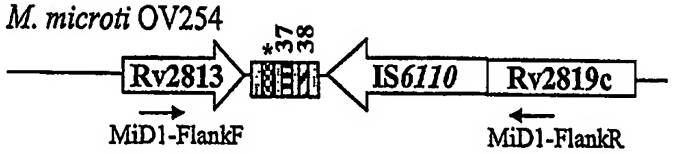


FIGURE 8A

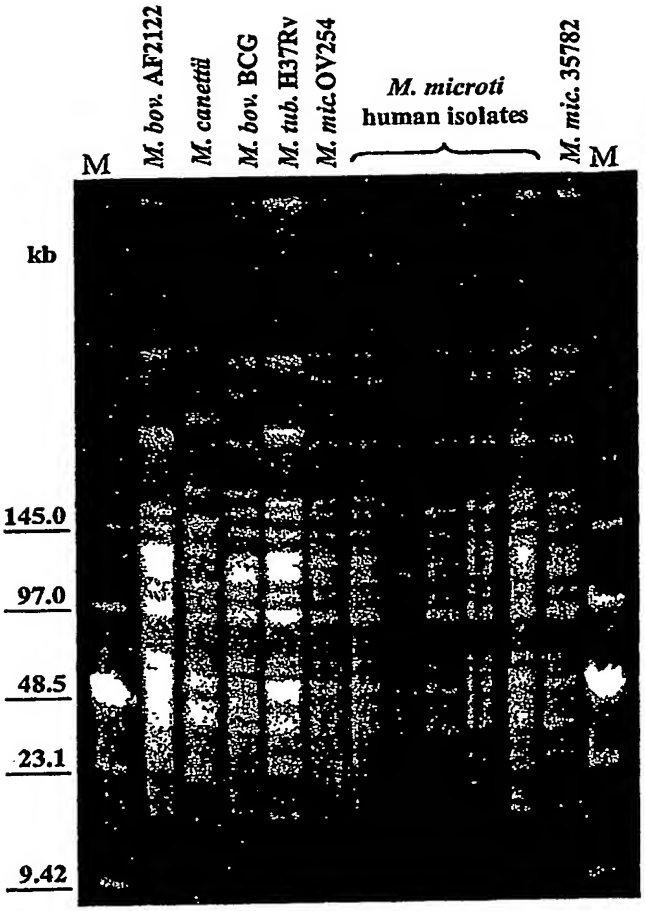


FIGURE 8B

FIGURE 8C

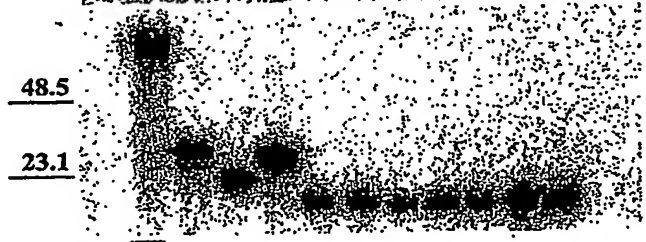
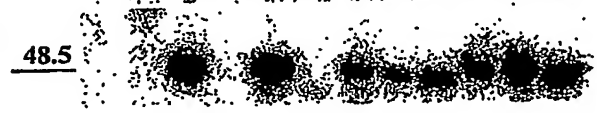


FIGURE 8D



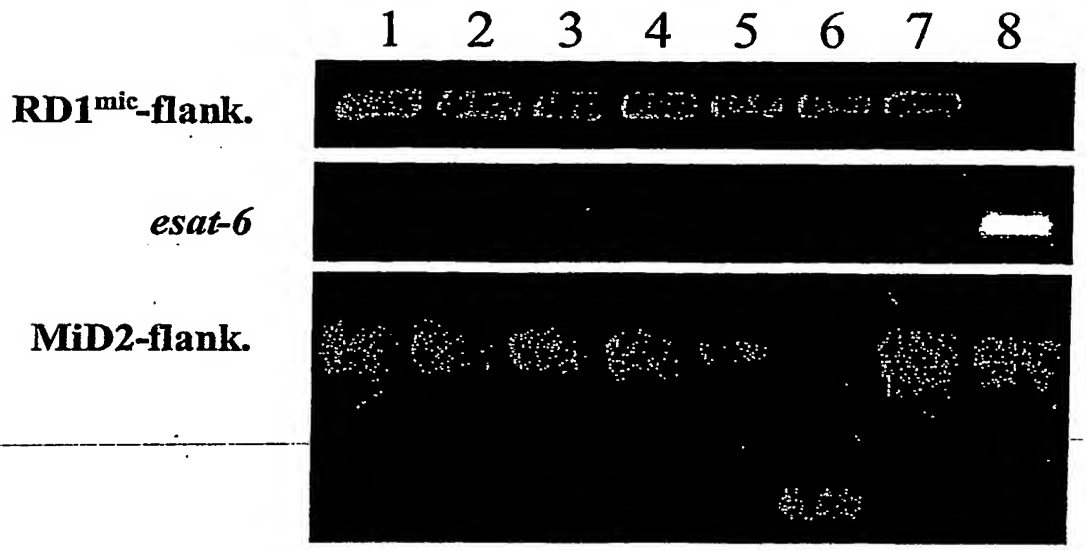


FIGURE 9

SEQUENCE LISTING PROVISOIRE

RD1-AP34 (a 3909 bp fragment of the M. tuberculosis H37Rv genome)

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ggctctaga

PE coding sequence (SEQ ID No 2)

atggaaaaaatgtcacatgatccgatcgctgccgacattggcacgcaagtgagcgacaacgctctgcacggcgtg
acggccggctcgacggcgctgacgtcggtgaccgggctggttcccgcgggggccgatgaggtctccgcccaagcg
gcgacggcgttcacatcggagggcatccaattgctggcttccaatgcatcggcccaagaccagctccaccgtgcg
ggcgaagcggtccaggacgtcgcccgcacctattcgcaaatcgacgacggcgccgccggcgtcttcgccgaa

PPE coding sequence (SEQ ID No 3)

atgctgtggcacgcaatgccaccggagctaaataccgcacggctgatggccggcgcgggtccggctccaatgctt
gcggcggccgcgggatggcagacgctttcggcggctctggacgctcaggccgtcgagttgaccgcgcgcctgaac
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CFP-10 coding sequence (SEQ ID No 4)

atggcagagatgaagaccgatgccgctaccctcgcgcaggaggcaggtaatttcgagcggatctccggcgacctg
aaaacccagatcgaccaggtggagtcgacggcaggttcgttgcagggccagtggcgcggcgcggcggggacggcc
gcccaggccgcggtggtgcgcttccaagaagcagccaataagcagaagcaggaactcgacgagatctcgacgaat
attcgtcaggccggcgtccaatactcgagggccgacgaggagcagcagcaggcgctgtcctcgcaaatgggcttc

ESAT-6 coding sequence (SEQ ID No 5)

Atgacagagcagcagtggaatttcgcgggtatcgaggccgcggcaagcgcaatccagggaaatgtcacgtccatt
cattccctccttgacgaggggaagcagtccctgaccaagctcgcagcggcctggggcggtagcggttcggaggcg
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atcagcgaagccggtcaggcaatggcttcgaccgaaggcaacgtcactgggatgttcgca

CFP-10 + ESAT-6 (SEQ ID No 6)

atggcagagatgaagaccgatgccgctaccctcgcgcaggaggcaggtaatttcgagcggat
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aacgtcactgggatgttcgca

Primer SP6-BAC1
AGTTAGCTCACTCATTAGGCA (SEQ ID No7)

Primer T7-BAC1
GGATGTGCTGCAAGGCGATTA (SEQ ID No8)

primer esat-6F
GTCACGTCCATTCATTCCCT (SEQ ID No 9);

primer esat-6R
ATCCCAGTGACGTTGCCTT) (SEQ ID No 10),

primer RD1^{mic} flanking region F
GCAGTGCAAAGGTGCAGATA (SEQ ID No 11);

primer RD1^{mic} flanking region R
GATTGAGACACTTGCCACGA (SEQ ID No 12)

primer plcA.int.F
CAAGTTGGGTCTGGTCGAAT (SEQ ID No 13)

primer plcA.int.R
GCTACCCAAGGTCTCCTGGT (SEQ ID No 14))

Sequences at the junction RD1^{mic}
CAAGACGAGGTTGTAAAACCTCGACGCAGGATCGGCGATGAAATGCCAGTCGGCGTCGCTGAGCGCGCGCTGCGC
CGAGTCCCATTTTGTCGCTGATTTGTTTGAACAGCGACGAACCGGTGTTGAAAATGTCGCCTGGGTCGGGGATTC
CCT (SEQ ID No 15)

primer RD5^{mic} flanking region F
GAATGCCGACGTCATATCG (SEQ ID No 16)

primer RD5^{mic} flanking region R
CGGCCACTGAGTTCGATTAT (SEQ ID No 17)

Sequence at the junction RD5^{mic}
CCTCGATGAACCACCTGACATGACCCCATCCTTTCCAAGAACTGGAGTCTCCGGACATGCCGGGGCGGTTCA*CTG*
CCCCAGGTGTCCTGGGTCGTTCCGTTGACCGTCGAGTCCGAACATCCGTCATTCCCGGTGGCAGTCGGTGCGGTG
AC (SEQ ID No 18)

primer MiD1 flanking region F
CAGCCAACACCAAGTAGACG (SEQ ID No 19)

primer MiD1 flanking region R
TCTACCTGCAGTCGCTTGTG (SEQ ID No 20)

Sequence at the junction MiD1
CACCTGACATGACCCCATCCTTTCCAAGAACTGGAGTCTCCGGACATGCCGGGGCGGTTCAGGGACATTCATGTC
CATCTTCTGGCAGATCAGCAGATCGCTTGTTCTCAGTGCAGGTGAGTC (SEQ ID No 21)

primer MiD2 flanking region R
GTCCATCGAGGATGTCGAGT (SEQ ID No 22)

primer MiD2 flanking region L
CTAGGCCATTCCGTTGTCTG (SEQ ID No 23)

Sequence at the junction MiD2
GCTGCCTACTACGCTCAACGCCAGAGACCAGCCGCCGGCTGAGGTCTCAGATCAGAGAGTCTCCGGACTCACCGG
GGCGGTTCA*TAAAGGCTTCGAGACCGGACGGGCTGTAGGTTCCTCAACTGTGTGGCGGATGGTCTGAGCACTTAA*
C (SEQ ID No 24)

primer MiD3 flanking region R
GGCGACGCCATTTCC (SEQ ID No 25)

primer MiD3 flanking region L
AACTGTCGGGCTTGCTCTT (SEQ ID No 26)

Sequence at the junction MiD3
TGGCGCCGGCACCTCCGTTGCCACCGTTGCCGCCGCTGGTGGGCGCGGTGCCGTTCGCCCCGGCCGAACCGTTCA
GGGCCGGGTTC*GCCCTCAGCCGCTAAACACGCCGACCAAGATCAACGAGCTACCTGCCCGGTCAAGGTTGAAGAG*
CCCCCATATCAGCAAGGGCCCGGTGTCGGCG (SEQ ID No 27)